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Robert M. Winslow, M.D.

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• VIII ISBS •

Eighth International Symposium on Blood Substitutes

November 9-11, 2000

San Diego, California, USA

SYLLABUS

Sponsored by

Department of Bioengineering,
University of California, San Diego

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The International Society for Artificial Cells,
Blood Substitutes and Immobilization Biotechnology

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National Heart, Lung, and Blood Institute,
The National Institutes of Health

VIIIth International Symposium on Blood Substitutes

November 8-11, 2000

San Diego, California

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Program and Abstracts

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Conference Chairmen and Organizing Committee

Robert M. Winslow, M.D., *Conference President*

Eishun Tsuchida, Ph.D.

Marcos Intaglietta, Ph.D.

Thomas M.S. Chang, M.D., Ph.D.

Scientific Committee

Robert M. Winslow, M.D.

Kim D. Vandegriff, Ph.D.

Eishun Tsuchida, Ph.D.

Amy Tsai, Ph.D.

Peter Keipert, Ph.D.

Marcos Intaglietta, Ph.D.

Thomas M.S. Chang, M.D., Ph.D.

Abdu Alayash, Ph.D.

Plenary Speakers

Robert M. Winslow, MD

President, Sangart, Inc.
11199 Sorrento Valley Road, Suite L
San Diego, CA 92121
rwinslow@sangart.com

H. Frankin Bunn, M.D.

Professor of Medicine, Harvard Medical School
Division of Hematology
Brigham & Women's Hospital
221 Longwood Avenue
LMRC-2, Room 223
Boston, MA 02115
bunn@calvin.bwh.harvard.edu

Eishun Tsuchida, Ph.D.

Professor of Polymer Chemistry
Waseda University
3-4 Ohkubo, Shinjuku-ku
Tokyo, Japan 169-8555
Japan
eishun@mn.waseda.ac.jp

John Frangos, Ph.D.

Professor of Bioengineering
Dept. of Bioengin, 0412
University of California, San Diego
9500 Gilman Drive,
La Jolla, CA 92093
frangos@bioeng.ucsd.edu

Abdu Alayash, Ph.D.

Center for Biologics, Evaluation
and Research
Food and Drug Administration
8800 Rockville Pike
Bethesda, MD, 20892
alayash@cber.fda.gov

Marcos Intaglietta, Ph.D.

Professor of Bioengineering
Dept. of Bioengineering, 5608
University of California, San
Diego
9500 Gilman Drive,
La Jolla, CA 92093
mintaglietta@ucsd.edu

Paul Johnson, Ph.D.

Professor of Bioengineering
Dept. of Bioengin, 0412
University of California, San
Diego
9500 Gilman Drive,
La Jolla, CA 92093
pjohnson@bioeng.ucsd.edu

Thomas M.S. Chang, M.D., Ph.D.

Professor of Physiology and
Medicine
McGill University
3655 Drummond Street
Montreal, Quebec, CANADA
H3G 1Y6
artcell@physio.mcgill.ca

Welcome from the Symposium President

Welcome to San Diego and the VIIIth ISBS. It is an honor and pleasure to have participated in organizing this year's Symposium, and all of us on the organizing committee are looking forward to 3 days filled with exciting science as well as renewal of friendships. We are also very excited about the number of new names among the registrants and authors/presenters, which demonstrates that "blood substitutes" has become a field of investigation with its own identity.

I want to personally recognize the outstanding contributions of my co-Chairmen. Professor Chang is recognized as the "grandfather" of blood substitutes, as demonstrated by his over 40 years of work in the field. Professor Tsuchida has been the scientific and opinion leader in Japan for decades, as has Professor Intaglietta in the US. I owe a great deal to all three of these scientists, as do all of us who are attending the VIIIth ISBS.

As always, putting on an international scientific congress is a team effort. Shirley Kolkey and her staff at Complete Conference Management have been handling all the details of the Congress site, hotel arrangements and the many details including choices for our coffee breaks and breakfasts. Their work began almost a year ago, and includes interacting with many of the speakers and attendees to meet many special requirements and, sometimes, demands. As always, their work has been outstanding.

In keeping with the digital age, we have made extensive use of the Internet this year, and we are especially indebted to Renee Schad for designing, implementing and updating our website, www.viii-isbs.com. Availability of the abstract forms and the electronic submission of abstracts have saved countless hours and has made the Congress and its news available worldwide for the first time to anyone with Internet access.

Finally, I want to express special thanks to my colleagues at Sangart, Carrie Hoopes, in particular, who have spent countless hours working on this abstract book and taking care of the many details of planning the program. Of course, all members of the Scientific Committee were of great assistance in making key decisions about the program, and its emphasis. Working with Peter Keipert, Abdu Alayash, Kim Vandegriff and Amy Tsai has been a real pleasure.

Again, welcome to San Diego and the VIIIth ISBS!

Sincerely,



Robert M. Winslow, MD
Congress President

VIIIth ISBS
Oral Presentations

Thursday November 9		Friday November 10		Saturday November 11	
830	Continental Breakfast	830	Continental Breakfast	830	Continental Breakfast
900	P1 Winslow	900	II-6 Vandegriff	900	P7 Intaglietta
930	P2 Bunn	920	II-7 Hsia	930	P8 Johnson
1010	Break	940	II-8 McDonagh	1000	Break
1030	I-1 Hirsch	1000	II-9 Biro	1020	V-1 Caron
1050	I-2 Fabry	1020	II-10 Feola	1035	V-2 Cheung
1110	I-3 Manjula	1040	Break	1050	V-3 Kerger
1130	I-4 Kramer	1100	P4 Tsuchida	1105	V-4 Kim
1150	I-5 Kjellstrom	1130	P5 Frangos	1120	V-5 Kingma
1210	Lunch	1200	Lunch	1135	V-6 Pittman
1400	II-1 Lowe	1330	III-1 Levin	1150	V-7 Sakai
1420	II-2 Keipert	1345	III-2 Nishiya	1205	Lunch
1440	II-3 Lemon	1400	III-3 Fischer	1330	P9 Chang
1500	II-4 Burhop	1415	III-4 Tablin	1400	VI-1 Nelson
1520	II-5 Carmichael	1430	III-5 Verhoeven	1415	VI-2 Peng
1540	Poster viewing Wine and Cheese	1445	discussion	1430	VI-3 Lundgren
		1500	Break	1445	VI-4 Acharya
		1530	P6 Alayash	1500	VI-5 Simoni
		1600	IV-1 Baldwin	1515	break
		1620	IV-2 D'Agnillo	1545	VI-6 Takeoka
		1640	IV-3 Panter	1600	VI-7 Wu
		1700	IV-4 Caron	1615	VI-8 Komatsu
				1630	VI-9 Tsuneshige
				1645	Conclusion
				1800	Reception
				1845	Dinner

Thursday, November 9

- P1 900 Winslow, R.M. BLOOD SUBSTITUTES: CURRENT STATUS AND NEW CHALLENGES
- P2 930 Bunn, F.H. OXYGEN SENSING AND OXYGEN-DEPENDENT GENE EXPRESSION

Free Communications: Chemistry and Physiology (Winslow)

- I-1 1030 Hirsch, R.E. THE INTRINSIC STABILITY OF LIGANDED HEMOGLOBINS IS DESTABILIZED BY CHLORIDE: ROLE OF SITE-SPECIFIC INTRAMOLECULAR MICRODOMAINS
- I-2 1050 Fabry, M.E. ADMINISTRATION OF FLUOROCARBON EMULSION REDUCES DEOXYHEMOGLOBIN IN LIVER AND KIDNEY OF A SICKLE TRANSGENIC MOUSE MODEL
- I-3 1110 Manjula, B.N. SITE SPECIFIC SURFACE DECORATION OF HEMOGLOBIN-A WITH POLYETHYLENE GLYCOL: CORRELATION BETWEEN INCREASED HYDRODYNAMIC VOLUME AND MASS OF PEG CONJUGATED
- I-4 1130 Kramer, G.C. HYPERONCOTIC, HYPEROSMOTIC AND HYPEROSMOTIC/HYPERONCOTIC HEMOGLOBIN SOLUTIONS
- I-5 1150 Kjellstrom, B.T. COMPLETE CIRCULATORY RESTORATION USING HEMOSPAN™ IN RESUSCITATION OF HEMORRHAGIC SHOCK IN PIGS

Free Communications: Clinical Development (Kjellstrom)

- II-1 1400 Lowe, K.C. CURRENT PERCEIVED RISKS OF TRANSFUSION IN THE UK AND RELEVANCE TO THE FUTURE ACCEPTANCE OF BLOOD SUBSTITUTES
- II-2 1420 Keipert, P.E. RECENT PROGRESS IN THE CLINICAL DEVELOPMENT OF *OXYGENT*™ AS AN INTRAVENOUS OXYGEN CARRIER FOR USE IN ELECTIVE SURGERY
- II-3 1440 Lemon, D. DESIGN OF SECOND GENERATION RECOMBINANT HEMOGLOBIN: MINIMIZING NITRIC OXIDE SCAVENGING AND VASOACTIVITY WHILE MAINTAINING EFFICACY
- II-4 1500 Burhop, K.E. HEMOGLOBIN-INDUCED MYOCARDIAL LESIONS
- II-5 1520 Carmichael, L. PHASE III CLINICAL TRIAL OF HEMOLINK™ IN CONJUNCTION WITH INTRAOPERATIVE AUTOLOGOUS DONATION (IAD) IN CARDIAC SURGICAL PATIENTS

Friday, November 10

Free Communications: Clinical Development (Kjellstrom)

II-6	900	Vandegriff, K.D.	HEMOSPAN™, A NON-HYPERTENSIVE, HIGH BLOOD FLOW RED CELL SUBSTITUTE
II-7	920	Hsia, C.J.C.	POLYNITROXYL HEMOGLOBIN (PNH): A NEW GENERATION RED CELL SUBSTITUTE WITH VASODILATION
II-8	940	McDonagh, P.F.	PERFLUBRON EMULSION REDUCES INFLAMMATION DURING EXTRACORPOREAL CIRCULATION
II-9	1000	Biro, G.	A 14-DAY INTRAVENOUS INFUSION TOXICOLOGY STUDY OF HEMOLINK™ (HLK) IN SPRAGUE-DAWLEY RATS
II-10	1020	Feola, M.	PRECLINICAL AND CLINICAL EXPERIENCE WITH A NOVEL HEMOGLOBIN ADENOSINE - GLUTATHIONE BASED RED CELL SUBSTITUTE
P4	1100	Tsuchida, E.	RECENT PROGRESS OF ARTIFICIAL BLOOD PROJECT AND NOVEL PRODUCTS
P5	1130	Frangos, J.	MECHANISMS OF FLOW INDUCED NO PRODUCTION

Minisymposium: Platelet Substitutes (Levin)

III-1	1330	Levin, J.	PLATELET SUBSTITUTES: AN OVERVIEW
III-2	1345	Nishiya, T.	ADHESIVE PROPERTIES OF LIPOSOMES CARRYING RECOMBINANT GPIIb/IIIa AND/OR GPIb UNDER FLOW CONDITIONS
III-3	1400	Fischer, T.H.	INTRACELLULAR STIMULUS-RESPONSE COUPLING AND POSITIVE FEEDBACK AMPLIFICATION OF HEMOSTATIC FUNCTIONS WITH REHYDRATED, LYOPHILIZED PLATELETS
III-4	1415	Tablin, F.	THE SIGNIFICANCE OF TEMPERATURE FOR PLATELET STORAGE AND THE DEVELOPMENT OF PLATELET SUBSTITUTES
III-5	1430	Verhoeven, A.	MECHANISM OF ACTION OF INFUSIBLE PLATELET MEMBRANES

Minisymposium: Oxidative Mechanisms (Alayash)

P6	1530	Alayash, A.I.	OXIDATIVE MECHANISMS OF HEMOGLOBIN-BASED BLOOD SUBSTITUTES
IV-1	1600	Baldwin, A.L.	SELENIUM REDUCES HEMOGLOBIN-INDUCED EPITHELIAL DAMAGE TO INTESTINAL MUCOSA
IV-2	1620	D'Agnillo, F.	REDOX REACTIONS OF HEMOGLOBIN ALTERS THIOL LEVELS AND THE MODE OF ENDOTHELIAL CELL DEATH
IV-3	1640	Panter, S.S.	HEMOGLOBIN-DEPENDENT NEUROTOXICITY
IV-4	1700	Caron, A.	EFFECTS OF DEX-BTC-Hb AND $\alpha\alpha$ -Hb ON HUMAN AORTIC ENDOTHELIAL CELL FUNCTIONS <i>IN VITRO</i>

Saturday, November 11

- P7 900 Intaglietta, M. MICROVASCULAR, MECHANICAL & CELLULAR BASIS FOR EFFECTIVE BLOOD SUBSTITUTES
- P8 930 Johnson, P.C. OXYGEN AND BLOOD FLOW REGULATION: THE SEARCH FOR THE MISSING LINK

Free Communications: Microcirculation and Vasoactivity (Intaglietta)

- V-1 1020 Caron, A. COMPARATIVE EFFECTS OF CROSS-LINKED, CONJUGATED AND POLYMERIZED HEMOGLOBINS ON HEMODYNAMICS AND BLOOD VISCOSITY AFTER MODERATE HEMODILUTION IN ANESTHETIZED RABBITS
- V-2 1035 Cheung, A.T.W. THE EFFECTS OF HEMOGLOBIN GLUTAMER-200 [BOVINE] ON THE MICROCIRCULATION IN A CANINE HYPOVOLEMIA MODEL: AN INTRAVITAL MICROSCOPY STUDY
- V-3 1050 Kerger, H. SYSTEMIC AND MICROCIRCULATORY EFFECTS OF AUTOLOGOUS WHOLE BLOOD TRANSFUSION IN SEVERE 4-HOUR HEMORRHAGIC SHOCK
- V-4 1105 Kim, H.W. DOES THIOL PLAY A ROLE IN HEMOGLOBIN MEDIATED VASOACTIVITY IN THE ISOLATED RAT THORACIC AORTA?
- V-5 1120 Kingma, J.G. HEMODILUTION WITH HEMOLINK™ DOES NOT ADVERSELY AFFECT LV HEMODYNAMICS OR BLOOD FLOW DISTRIBUTION IN ANESTHETIZED DOGS
- V-6 1135 Pittman, R.N. EFFECTS OF OXYGENT™ ON BLOOD OXYGENATION IN CAPILLARIES FOLLOWING ISOVOLEMIC HEMODILUTION
- V-7 1150 Sakai, H. MOLECULAR DIMENSIONS OF Hb-BASED O₂ CARRIERS DETERMINE CONSTRICTION OF RESISTANCE ARTERIES AND HYPERTENSION
- P9 1330 Chang, T.M.S. PRESENT STATUS OF NEW GENERATION OF HEMOGLOBIN PRODUCTS

Free Communications: Novel Products and Applications (Chang)

- VI-1 1400 Nelson, J.W. TREATMENT OF SEVERE DECOMPRESSION SICKNESS IN SWINE WITH OXYGENT™, A PERFLUOROCARBON EMULSION
- VI-2 1415 Peng, C.-A. LESS PHAGOCYTOSIS OF FLUOROCARBON EMULSION MADE BY FLUOROALKYLATED POLYETHYLENE GLYCOL
- VI-3 1430 Lundgren, C. PERFLUOROCARBON-STABILIZED INTRAVASCULAR MICROBUBBLES: AN ULTRA-EFFECTIVE MODE OF OXYGEN DELIVERY
- VI-4 1445 Acharya, A.S. BIS MALEIDOPHENYL-PEG REAGENTS FOR INTERMOLECULAR CROSS-BRIDGING (SIZE ENHANCEMENT) OF HEMOGLOBIN A
- VI-5 1500 Simoni, J. ATTENUATION OF HEMOGLOBIN (Hb) NEUROTOXIC POTENTIAL BY CROSS-LINKING WITH ADENOSINE AND CONJUGATION WITH REDUCED GLUTATHIONE (GSH)
- VI-6 1545 Takeoka, S. SYNTHESIS AND PHYSICOCHEMICAL CHARACTERIZATION OF Hb-BASED O₂ CARRIERS: COMPARISON BETWEEN CELLULAR AND ACELLULAR TYPES
- VI-7 1600 Wu, Y.P. ELECTROCHEMICAL STUDIES OF AN ALBUMIN-HEME HYBRID AS OXYGEN-CARRING HEMOPROTEIN
- VI-8 1615 Komatsu, T. NO-BINDING PROPERTIES OF RECOMBINANT HUMAN SERUM ALBUMIN INCORPORATING SYNTHETIC HEME (ALBUMIN-HEME)
- VI-9 1630 Tsuneshige, A. HUMAN ERYTHROCYTES CONTAINING EXCLUSIVELY α -NITROSYL HEMOGLOBIN: A PROMISING BLOOD TRANSFUSANT CANDIDATE

Abstracts of Oral Presentations

P-1. BLOOD SUBSTITUTES: CURRENT STATUS AND NEW CHALLENGES

R.M. Winslow. Sangart, Inc., San Diego CA 92121

Although "blood substitutes" have been sought for decades, almost all of the current products under development today arose out of the perceived dangers of blood transfusion in the early 1980's. While hemoglobin- and perfluorocarbon-based products are very near clinical approval, impressive strides have been made by the Blood Bank community in assuring the safety of stored blood. Nevertheless significant limitations still exist, including increasing shortages caused by rising demand, dwindling supply and rising cost. One challenge, therefore, is to develop products that can be produced in large scale at a cost that is competitive with blood. New synthetic strategies should be focused on cost from the very beginning of their development, and better ways need to be found to join peer-reviewed research efforts with commercial production early in the development cycle. Most of the current products were conceived at a time when very little was known about how tissue is perfused at the level of the microcirculation, and how local flow is regulated by the many vasoactive compounds and by the gases NO and O₂. The considerable research that has accompanied development of current products has led to an exciting new understanding of how oxygen is delivered and waste materials are removed in the microcirculation. Some of the results, to be presented at this symposium, are highly controversial and counter-intuitive. Therefore another challenge is to tailor cell-free products to the microenvironment of tissue oxygenation, rather than rely on old assumptions about their physical properties. Such efforts are difficult because the research needed is necessarily multi-disciplinary and sometimes expensive and tedious. Finally, many products have progressed through preclinical and even early clinical development, only to arrive at the threshold of licensure to be entangled in complicated clinical trials which are difficult to interpret. Therefore, an additional major challenge is to work with regulatory authorities to develop clinical trial strategies that will ensure demonstration of safety and efficacy in a manner that is both timely and cost-effective.

P-2. OXYGEN SENSING AND OXYGEN-DEPENDENT GENE EXPRESSION

Zhu, H., Jackson, T. and Bunn, F.H. Division of Hematology, Brigham and Women's Hospital, Harvard Medical School, Boston MA 02115

A growing number of physiologically relevant genes are regulated in response to changes in intracellular oxygen tension. It is likely that cells from a wide variety of tissues share a common mechanism of oxygen sensing and signal transduction leading to the activation of the transcription factor hypoxia inducible factor 1 (HIF-1). There is growing, albeit indirect, evidence that the oxygen sensor is a flavoheme protein and that the signal transduction pathway involves changes in the level of intracellular reactive oxygen intermediates (ROI). We have recently cloned a novel fusion protein consisting of an N-terminal domain with homology to cytochrome b5 and a C-terminal domain homologous to cytochrome b5 reductase. The expressed protein is a cyanide insensitive NADPH oxidase and therefore a candidate for the oxygen sensor. This flavoheme protein is widely expressed in cell lines and tissues, with localization in the perinuclear space. It utilizes either NADH or NADPH to convert oxygen to superoxide with a half maximal velocity at 2% oxygen. The activation of HIF-1 by hypoxia is likely to depend upon ROI-dependent rescue of its α -subunit from oxygen-dependent degradation in the proteasome, allowing it to form a heterodimer with HIF-1 β (ARNT), which then translocates to the nucleus, enabling transcription of genes whose cis-acting elements contain cognate hypoxia response elements.

I-1. THE INTRINSIC STABILITY OF LIGANDED HEMOGLOBINS IS DESTABILIZED BY CHLORIDE: ROLE OF SITE-SPECIFIC INTRAMOLECULAR MICRODOMAINS

Q.Y. Chen¹, C. Bonaventura², J.M. Friedman¹, R.L. Nagel¹, and R.E. Hirsch¹. ¹Albert Einstein College of Medicine, Bronx, NY 10461, ²Duke University Marine Laboratory, Beaufort, NC 28516

Chloride is an important regulator of hemoglobin (Hb) function. Chloride stabilizes T-state Hb (Bonaventura et al., 1998). In contrast, we now show that the intrinsic stability of R-state (Oxy or CO) hemoglobins is destabilized by low levels of chloride (Hepes buffer). Recently, some of us have demonstrated structural perturbations in the $\beta 6$ variants, HbC ($\beta 6$ Glu \rightarrow Lys) and HbS ($\beta 6$ Glu \rightarrow Val), distal to the site of mutation involving the A-helix and the diphosphoglycerate (DPG) binding pocket (Hirsch et al., 1999). The effect of chloride on the oxidation and mechanical precipitability of R-state HbA, HbC, and HbS is compared. Using front-face optics, the extent of oxidation is determined by the intensity change of the fluorescent oxidation product (ex. 321nm, em.max. 465nm) (Rifkind et al., 1998). Mechanical precipitability is determined according to the method of Asakura et al. (1974) and was previously demonstrated to parallel the extended uncoiling of HbS at an air-water interface (Hirsch et al., 1980). Results show that HbC exhibits a propensity to oxidize and precipitate less than HbS but greater than HbA. 0.1M chloride (Hepes buffer) promotes oxidation and mechanical precipitation of HbS>HbC>HbA. Chloride titration (0-1.5mM) of R-state HbA,C,S monitored by intrinsic fluorescence shows a decrease in the intrinsic fluorescence in the order HbA>HbS>HbC. Relative front-face fluorescence measurements of fluorescein bound at $\beta 93$ Cys as a function of chloride titration (0-0.8mM) suggest a difference in the $\beta 93$ side chain orientation of these $\beta 6$ mutants compared to HbA. Chloride (0-0.3M) displaces HPT (the fluorescent DPG analog) from the liganded hemoglobin central cavity in the order HbC>HbS>HbA. These results also indicate (1) a signaling pathway(s) at the chloride binding site that involves the β -chain A-helix, central cavity, and heme microenvironment, and (2) this pathway appears to be altered in liganded forms of the $\beta 6$ mutants.

I-2. ADMINISTRATION OF FLUOROCARBON EMULSION REDUCES DEOXYHEMOGLOBIN IN LIVER AND KIDNEY OF A SICKLE TRANSGENIC MOUSE MODEL

M.E. Fabry¹, M.D. Does², S.M. Suzuka¹, and R.L. Nagel¹. ¹Dept Medicine, Albert Einstein College of Medicine, Bronx, NY and ²Department of Diagnostic Radiology, Yale Univ School of Medicine, New Haven, CT

Poorly oxygenated tissues are at increased risk of vaso-occlusion in the presence of polymerizable hemoglobins. We previously detected deoxyHb in kidney and liver of the sickle cell disease mouse model, S+S-Antilles (JCI 98:2450-2455, 1996) by use of blood oxygen level dependent-magnetic resonance imaging (BOLD-MRI) in which image intensity is compared in tissues while the animals first breathe room air and then 100% O₂. If elevated deoxyHb is due to reduction of flow or partial obstruction by sickled cells, then infusion of oxygen carrying material with small particle size (<0.4 μ) such as a perfluorocarbon emulsion could improve oxygenation and flow. A perfluorocarbon emulsion comprised primarily of Perflubron® (PFCE) provided by Alliance Pharmaceutical Corp was administered to S+S-Ant mice at volumes equivalent to 5%, 10%, and 20% of blood volume (BV) by tail vein and BOLD MRI images were obtained. The intensity change was compared to that seen in C57 mice and C57 mice injected with 10% BV of PFCE. We found that PFCE at 10% of BV results in a reduction in the change of image intensity to that observed in controls (C57BL). Since the excess % change in signal intensity is proportional to the deoxyHb present, we conclude that infusion of PFCE results in reduction of deoxyHb in S+S-Antilles mouse kidney and liver. Infusion of PFCE may reduce risk of extending and/or alleviate sickle cell vaso-occlusion during sickle cell painful crisis or sickle liver crisis.

#	Mouse	N	PFCE	% MRI change liver	% MRI change kidney
1	C57BL	3	0	24 \pm 7%	9 \pm 3%
2	S+S-Ant	3	0	55 \pm 5%, P ₁₂ <0.02	19 \pm 2%, P ₁₂ <0.05
3	S+S-Ant	1	5% BV	24%	5%
4	S+S-Ant	2	10% BV	33.5 \pm 4%, P ₂₄ <0.04	10 \pm 1%, P ₂₄ <0.02
5	S+S-Ant	1	20% BV	25%	3%

I-3. SITE-SPECIFIC SURFACE DECORATION OF HEMOGLOBIN-A WITH POLYETHYLENE GLYCOL: CORRELATION BETWEEN INCREASED HYDRODYNAMIC VOLUME AND MASS OF PEG CONJUGATED

B.N. Manjula¹, R. Upadhyay¹, A. Nemkal¹, P.K. Smith², K.D. Vandegriff³, R.M. Winslow³, J.M. Friedman¹, and A.S. Acharya¹. ¹Albert Einstein College of Medicine, Bronx, NY, ²BioAffinity Systems, Rockford, IL, and ³Sangart, San Diego, CA

Generation of Hb with either reduced NO binding or enhanced molecular size are two approaches advanced to overcome the vasoactivity of Hb. A recent study has shown that PEGylation of the ϵ -amino groups of Hb, which increases its viscosity, also neutralizes its vasoactivity. In an attempt to understand the molecular origin of this neutralization, new PEGylation protocols have been developed to achieve a site-specific attachment of PEG to Cys-93(ϵ) by maleimide chemistry or to the γ -amino groups by reductive alkylation chemistry. Homogenous preparations of Hb carrying two copies of PEG chains of 5K, 10K and 20K at its Cys-93(ϵ) have been prepared [(SP-PEG-5K)₂-HbA, (SP-PEG-10K)₂-HbA and (SP-PEG-20K)₂-HbA (SP=succinimidophenyl)]. The studies of these products have established: (i) The surface decoration increases the hydrodynamic volume of Hb; the increase in the apparent molecular size (hydrodynamic volume) on surface decoration is considerably larger than what is calculated from the actual mass of the conjugated PEG-chains; PEG increases the apparent molecular size by about eight times its actual mass. (ii) The size enhancement correlates well with the mass of the PEG chain conjugated. (iii) The molecular radii of (SP-PEG-5K)₂-HbA, (SP-PEG-10K)₂-HbA and (SP-PEG-20K)₂-HbA are 4.8, 5.7 and 5.9 respectively, as compared to 3.3 for control HbA. Thus, the molecular radius is not a direct correlate of the molecular size of PEG. (iv) The viscosity of the surface decorated Hb is sensitive to the mass of the conjugated PEG-chains. (v) O₂-affinity of all modified Hb is higher than that of HbA, but the mass of the PEG-chain has very little influence on the O₂ affinity. A homogeneous preparation of (Pr-PEG-20K)₂-HbA (Pr = Propyl) with conjugation of PEG on the γ -amino groups has also been generated. The O₂-affinity of (Pr-PEG-20K)₂-HbA is slightly lower than that of HbA and its hydrodynamic volume is comparable to that of (SP-PEG-20K)₂-HbA. Thus, the size enhancement of Hb on PEGylation is independent of the chemistry of conjugation but is dependent on the size of the PEG. These new classes of homogeneous surface-decorated Hb preparations can facilitate the delineation of the interplay of O₂-affinity and colligative properties of surface decorated Hb in the neutralization of the vasoactivity of acellular Hb.

I-4. HYPERONCOTIC, HYPEROSMOTIC AND HYPER-OSMOTIC/ HYPERONCOTIC HEMOGLOBIN SOLUTIONS

G.C. Kramer. Departments of Anesthesiology and Physiology, University of Texas Medical Branch, Galveston Texas 77555-0801

Combined hyperosmotic-hyperoncotic solutions for small volume resuscitation of hypovolemia and shock were first described in 1985-86. Early utility of this concept focused largely on combining hypertonic crystalloid (NaCl) with hyperoncotic colloid (dextran). However, research with different crystalloids (sodium acetate, glucose, sodium bicarbonate, and mannitol); different synthetic colloids (hetastarch); and synthetic and natural protein colloids (albumin, gelatin and hemoglobin) were also evaluated in animal experiments and mathematical models. The predominant physiologic effects of infused 7.5% NaCl / 6% dextran 70 (HSD) into hemorrhaged mammals, including humans, are volume expansion, reduced peripheral resistance and improved cardiac effectiveness. The advantages of making a hyperosmotic-oxygen carrier were further presented and evaluated with both perfluorocarbons and hemoglobins. In brief, HSD or HS-hetastarch corrects O₂ debt of circulatory shock by improving blood flow, but the resultant plasma volume expansion decreases hemoglobin concentration and blood O₂ content. Hemoglobin based oxygen carrier solutions (HBOC) were developed for their ability to improve the O₂ content of blood, but they have had varying effects on cardiac output and tissue perfusion. Many of the first generation hemoglobin based oxygen carriers reduced cardiac output in normovolemia, and increased cardiac output less than an equal volume of iso-oncotic colloid in hypovolemia. Most HBOC solutions are formulated to be hyperoncotic colloids in isotonic electrolyte solution. Such isotonic-hyperoncotic solutions are considered a disadvantage for a RBC substitute due to hemodilution of both endogenous and exogenous blood hemoglobin, but a hyperosmotic-hyperoncotic formulation may be an advantage for a small volume hyperosmotic-hyperoncotic hemoglobin or a perfluorocarbon solution. Despite theoretical advantages, animal studies of hyperosmotic-hyperoncotic HBOC suggest only limited added benefit compared to HSD. However, modeling suggests that more effective solutions can be formulated by optimizing the ratio of osmotic pressure to oncotic pressure, and maximizing the O₂ capacity of the modified Hb molecule.

I-5. COMPLETE CIRCULATORY RESTORATION USING HEMOSPAN™ IN RESUSCITATION OF HEMORRHAGIC SHOCK IN PIGS

B.T. Kjellström¹, D. Drobin¹, M. Intaglietta², J.M. Lohman³, K.D. Vandegriff³, R.M. Winslow³.

¹Department of Experimental Traumatology, Swedish Defense Research Establishment and Karolinska Institute at Söder Hospital Stockholm, Sweden; ²Department of Bioengineering, University of California, San Diego, CA, USA; ³ Sangart, Inc., San Diego, CA, USA

Hemospan™ (maleimide PEG-Hb) with novel physicochemical properties was developed to overcome detrimental blood flow reductions seen with previous hemoglobin-based solutions. Resuscitation with three different formulations of Hemospan™ was performed in 24 Ketamine anesthetized pigs subjected to a 50% hemorrhage, which causes a 50% mortality in unresuscitated animals. Six control animals were resuscitated with Pentaspan®. Resuscitation volumes were 70% of shed volume. All animals were instrumented for continuous registration of hemodynamic parameters. Blood samples were withdrawn for blood gas analysis, lactate, plasma hemoglobin concentration, hematocrit and electrolytes every 15 minutes. The observation period was 150 minutes posthemorrhage and all animals survived. Cardiac output decreased during hemorrhage but recovered similarly in all groups as did the systemic pressure following resuscitation. The systemic vascular resistance was reduced during infusion and tended to return to baseline values following resuscitation in the treatment groups. Small, parallel increases in pulmonary artery pressure and cardiac output in all treatment groups resulted in unchanged pulmonary vascular resistance. Unexpectedly, Hemospan™ caused a marked increase in oxygen extraction ratio and oxygen consumption that was contrasted by reductions in both parameters in the Pentaspan®-treated controls.

Hemospan™ showed high-flow properties not observed in earlier artificial cell-free blood preparations. Such properties should be a prerequisite for future clinical applications.

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II-1. CURRENT PERCEIVED RISKS OF TRANSFUSION IN THE UK AND RELEVANCE TO THE FUTURE ACCEPTANCE OF BLOOD SUBSTITUTES

K.C. Lowe¹, K. Farrell², V. James³, and E.M.P. Ferguson². ¹Schools of Life & Environmental Sciences and ²Psychological Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK & ³National Blood Service, Longley Lane, Sheffield S5 7JN, UK

While the biology of blood and its products is becoming increasingly better understood and the spectrum of transfusion options increasing, it is surprising how little research has focused on the public's perceptions of risk with regard to the receipt of blood and blood products. We do not know (i) what the public know about transfusion, (ii) how safe they perceive blood/blood products to be, and (iii) what different key groups [e.g. general practitioners (GPs) and journalists] know about blood transfusion and, crucially, how this might influence their own and other groups perception of risk associated with blood transfusion. This novel project has gathered data on the perception of risk and levels of knowledge associated with blood donation and transfusion (including blood substitutes) in UK adult blood donors, anaesthetists, GPs and healthcare journalists of both genders. A questionnaire survey was conducted from March-July 2000 involving (i) blood donors (n = 250), (ii) GPs (n = 88), (iii) anaesthetists (n = 143), (iv) and journalists (n = 20). Significant ($P < 0.001$) differences in knowledge base on blood and blood products were found between the sample groups, but there were no significant differences in the perception of risk associated with blood transfusion. The percentage of respondents who would prefer to receive their own blood, compared to donor blood or a suitable substitute ranged from 73-94%. When required to choose between donor blood or a blood substitute, there were significant ($P < 0.05$) differences between sample groups: anaesthetists and GPs preferred to receive a blood substitute (52-59%), whereas blood donors and journalists would prefer donated blood (74-93%). These findings have clear implications for the future development and implementation of modern transfusion options, including the use of blood substitutes as alternatives to donor blood.

II-2. RECENT PROGRESS IN THE CLINICAL DEVELOPMENT OF *OXYGENT*[™] AS AN INTRAVENOUS OXYGEN CARRIER FOR USE IN ELECTIVE SURGERY

P.E. Keipert. Alliance Pharmaceutical Corp., San Diego, CA 92121

Oxygent (60 percent w/v perflubron-based emulsion) is a temporary O₂ delivery agent ("blood substitute") in clinical development to reduce the need for donor blood transfusion in surgical patients. *Oxygent* is the only blood substitute manufactured from commercially available raw materials not derived from human or cow blood. It is provided as a ready-to-use sterile emulsion that is compatible with all blood types, and can be stored for up to two years. The use of *Oxygent* is intended to permit more effective collection of greater quantities of autologous blood just prior to start of surgery (Augmented-Acute Normovolemic Hemodilution), thereby allowing more patients to be their own donor without having to go through the more complex logistics associated with predonation. To date, >1,200 subjects have been enrolled in 18 completed clinical studies. Phase 2 studies in general surgery patients have demonstrated that *Oxygent* enhanced oxygenation status, and was significantly more effective than a unit of blood at reversing physiologic transfusion "triggers" (i.e., indicators for a blood transfusion) and delaying the need for subsequent blood transfusion. Recently, Alliance completed an international, Phase 3 study involving 492 general surgery patients at 34 centers in eight European countries. Preliminary analysis of efficacy data demonstrated that patients receiving *Oxygent* required significantly ($p=0.01$) fewer units of allogeneic blood. In the patient population targeted by this protocol (i.e., estimated blood loss >20 mL/kg), *Oxygent*-treatment resulted in a highly significant ($p<0.001$) reduction and avoidance of allogeneic blood compared to the control group. A complementary multicenter Phase 3 Transfusion Avoidance study is also underway in cardiac surgery patients undergoing coronary artery bypass grafting with cardiopulmonary bypass. This study, which will enroll 600 patients (primarily at centers in the U.S., with additional participation by sites in Canada and Europe), is expected to complete enrollment of patients in the first quarter of 2001.

II-3. DESIGN OF SECOND GENERATION RECOMBINANT HEMOGLOBIN: MINIMIZING NITRIC OXIDE SCAVENGING AND VASOACTIVITY WHILE MAINTAINING EFFICACY

M.P. Doyle, A.M. Armstrong, E.A. Brucker, T.J. Fattor, and D.D. Lemon. Baxter Hemoglobin Therapeutics, Boulder, CO 80301

Administration of extracellular hemoglobin-based oxygen carriers often increases blood pressure and causes other side effects. We believe that these effects are due primarily to scavenging of nitric oxide (NO) by oxy- and deoxyhemoglobin. Accordingly, we have engineered new hemoglobin variants that have lower intrinsic reactivity toward NO. Mutagenesis of the distal heme pockets of hemoglobin has produced many variants having a wide range of NO kinetics. We have previously shown that the magnitude of the blood pressure effect correlates directly with *in vitro* NO reactivity (D.H. Doherty, et al, Nature Biotechnology 16:672-676, 1998). More recently, using hemoglobin variants with low oxygen affinity, we have found that total peripheral resistance is also strongly dependent on intrinsic NO reactivity. We have produced and tested hemoglobin variants with native NO kinetics and p_{50} values ranging from 3-46 mm Hg. These results demonstrate that blood pressure and total peripheral resistance increases are independent of oxygen affinity. Thus, we have concluded that the fundamental mechanism of vasoconstriction is reaction of NO with oxy- and deoxyhemoglobin. Importantly, we were able to retain efficacy by optimizing oxygen-binding parameters in the context of minimal NO reactivity. This work has led to the selection of a second-generation candidate, currently in preclinical testing, based on recombinant hemoglobin having favorable NO scavenging and oxygen binding properties.

II-4. HEMOGLOBIN-INDUCED MYOCARDIAL LESIONS

K.E. Burhop, and T.E. Estep. Baxter Healthcare Corporation, Hemoglobin Therapeutics, Boulder, CO 80503

Over 100 preclinical studies in several small and large animal species were performed to evaluate the safety and efficacy of diaspirin crosslinked hemoglobin (DCLHb; Baxter Healthcare Corp.). During this preclinical evaluation of DCLHb, a pathological finding observed after DCLHb administration to certain species was myocardial lesions. These lesions were characterized by mild to moderate focal-to-multifocal myocardial degeneration and/or necrosis, generally affecting less than 3% of the total myocardium. The lesions were typically found 24 - 48 hours after single topload infusions of DCLHb into rhesus monkeys and pigs at doses as low as 200 or 700 mg/kg, respectively. Dogs, sheep and rats did not develop this pathology after single-dose administrations of DCLHb. The left ventricular myocardium near the base of the papillary muscle was the most severely affected region, followed by the intraventricular septum and then the right ventricle. The left and right atria were usually not affected. These lesions were also observed after infusion into pigs of either human or pig stroma-free hemoglobin. Process residuals or contaminants, specific crosslinking chemistry, processing steps or procedures, the infusion protocol utilized or the volume status of the animal were not responsible for inducing these lesions. Although increases in serum enzyme activities (AST, CK, LDH) were observed, isoenzyme analysis indicated a source other than myocardium. ECG analysis and echocardiography were not sensitive enough to detect these lesions. Polymerization reduced but did not eliminate the incidence and severity of the lesions. Infusion of hemoglobin solutions with reduced interaction rates with nitric oxide (NO) resulted in a significant decrease in the incidence and severity of these lesions, while administration of L-NAME, a NO synthase inhibitor, resulted in the appearance of lesions which were indistinguishable from those induced by hemoglobin. Overall, the presence of myocardial lesions represents a pathologic finding that must be considered during the testing and development of new HBOCs.

II-5. PHASE III CLINICAL TRIAL OF HEMOLINK™ IN CONJUNCTION WITH INTRAOPERATIVE AUTOLOGOUS DONATION (IAD) IN CARDIAC SURGICAL PATIENTS

F.J.L. Carmichael¹, G.P. Biro¹, D.C.H. Cheng², Toronto, Ontario, Canada ¹Hemosol Inc.,
²Toronto General Hospital, University Health Network

A clinical trial involving 299 patients undergoing coronary artery bypass grafting (CABG) surgery was completed in 24 medical centres in Canada and Britain. Patients who consented and satisfied inclusion criteria were randomly assigned (1:1) to receive either 750 ml Hemolink™ (o-raffinose cross-linked human hemoglobin), or 750 ml Pentaspan®, as partial replacement of an intraoperative autologous blood donation (IAD) of 500-2000mL, which was calculated to reduce the RBC hemoglobin to approximately 7 g/dL, at the beginning of cardio-pulmonary bypass. When on bypass, IAD blood was transfused if the hemoglobin fell below 6 g/dL, or if other clinical indications for an intraoperative transfusion were present. If all IAD blood was used, then allogeneic red blood cells (RBCs) were transfused if required. All IAD blood remaining after completion of the surgery was reinfused at that time. Transfusions of allogeneic RBCs were given if the transfusion trigger of 7 g/dL in the ICU, or 8 g/dL post-ICU was reached, or if otherwise clinically indicated. In all respects the local practice of anesthesia, surgery and post-surgical care was followed. Transfusions of IAD blood intraoperatively, and of allogeneic RBCs and other blood products given, were recorded during the initial hospitalization and the subsequent 6 to 8 week follow up period post-discharge. The actual IAD volume harvested was similar in the HLK and PS arms. Fifteen minutes after completion of the IAD procedure the whole blood hemoglobin concentration was 7.98 ± 1.04 and 6.89 ± 1.08 g/dL in the HLK and PS arms, respectively. This difference narrowed to 0.6 g/dl after 24 hours. Fewer patients randomized to the HLK arm than to the PS arm required: i) IAD transfusion intraoperatively, ii) allogeneic RBCs intra- and postoperatively, and iii) allogeneic non-RBC blood products. Those HLK patients who received allogeneic RBCs and blood products, received fewer units, on average.

II-6. HEMOSPAN™, A NON-HYPERTENSIVE, HIGH-BLOOD FLOW RED CELL SUBSTITUTE

K.D. Vandegriff¹, J.M. Lohman¹, A.G. Tsai², M. Intaglietta², D. Drobin³, B.T. Kjellström³, and R.M. Winslow¹. ¹Sangart, Inc., San Diego, CA, ²Department of Bioengineering, University of California, San Diego, CA, ³Swedish Defense Research Establishment and Karolinska Institute at the Söder Hospital, Stockholm, Sweden

Hemospan™ is a new red cell-based blood substitute product based on these design principles: 1) high O₂ affinity, 2) solution viscosity close to blood, 3) hyperoncoticity, and 4) high molecular volume:mass ratio. These principles were counterintuitive in the past, and the majority of acellular hemoglobin solutions designed 10-20 years ago had: 1) low O₂ affinity similar to red blood cells, 2) low viscosity similar to water, 3) iso-oncotic formulation, and 4) high [Hb]. As acellular solutions, these early products caused vasoconstriction, as evidenced by increased mean arterial blood pressure and lowered cardiac output. These physiological changes resulted in increased systemic vascular resistance, which is detrimental to tissue perfusion. Sangart has developed a human tetrameric surface-conjugated hemoglobin using maleimide polyethylene glycol chemistry. Pre-clinical studies have been conducted with Hemospan™ in: 1) hamsters for direct observation of the microcirculation, 2) rats for a surgical simulation of hemodilution followed by potentially lethal hemorrhage, and 3) swine for a hemorrhage/resuscitation protocol. Our studies show that Hemospan™ maintains good capillary blood flow during severe hemodilution in hamsters, insignificant changes in vascular resistance compared to baseline values and high O₂ consumption with low [Hb] following rapid resuscitation from hemorrhagic shock in swine. In our rat protocol, we observed 100% survival over 2 hrs after 50% hemodilution followed by severe hemorrhage; this protocol is lethal in non-hemodiluted controls and controls hemodiluted with Pentaspan®, matched for oncotic pressure and viscosity with the product but for not O₂ capacity. In summary, the novel physicochemical properties of Hemospan™ prevent the typical vascular response to cell-free hemoglobins and provide a promising new product for clinical development.

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II-7. POLYNITROXYL HEMOGLOBIN (PNH): A NEW GENERATION RED CELL SUBSTITUTE WITH VASODILATORY AND ANTIOXIDANT PROPERTIES

C.J.C. Hsia, Ph.D., SynZyme Technologies LLC, Irvine, CA 92618

Much effort has been directed toward the creation of an oxygen-carrying, volume expanding transfusion fluid to replace lost red cells in the hemorrhaged combat casualty. A number of first-generation hemoglobin (Hb)-based oxygen carriers (HBOCs) appear to be nearing approval for use in elective surgery. However, *vasoconstriction* and toxicity, possibly related to scavenging of nitric oxide (NO) by Hb and the pro-oxidant activity of Hb, have been attributed to the suspension of clinical development of HemAssist[®] and Optro[®]. Accordingly, concerns remain regarding the potential toxicity of most HBOCs currently in development. SynZyme Technologies aims to create a new type of red cell substitute comprising a *vasodilatory* Hb-based enzyme mimic with antioxidant and anti-inflammatory therapeutic activities in addition to its oxygen transport function. This is achieved by covalently linking a nitroxide moiety to a HBOC. This formulation is called polynitroxylated hemoglobin (PNH). Three characteristics of PNH suggest that this compound may have important advantages over both red blood cells and HBOCs for the resuscitation of combat casualties. First, PNH is a *vasodilator* which enhances blood flow and oxygen delivery to the tissues. Second, PNH is an *antioxidant*, which increases its resistance to auto-oxidation *in vivo*, leading to increased stability and decreased release of toxic heme and iron. Finally, in contrast with first-generation HBOCs, PNH possesses *anti-inflammatory activities*, which may enable it to prevent/treat oxidative ischemia, reperfusion and inflammatory injuries and post-reperfusion multiple organ failure (MOF) following severe hemorrhage and resuscitation. Results will be presented to show that in comparison with a control HBOC in a hemorrhagic shock and resuscitation model, PNH significantly improved hemodynamics, base deficit, and survival. In summary, PNH is a new red cell substitute with strong potential for dual use in military and civilian trauma care, where no comparable product is available to meet the need for prevention/treatment of oxidative reperfusion injury and post-ischemic MOF in the severely hemorrhaged casualty. (Supported in part by US Army Medical Research Contract No. ERMS#00110002 and Office of Naval Research Contract No. N-000-14-98-C-0140 and NIH SBIR Grants 1R43HL6582-01, 1R44HL66887-01, 1R43HL53860-01).

II-8. PERFLUBRON EMULSION REDUCES INFLAMMATION DURING EXTRACORPOREAL CIRCULATION

P.F. McDonagh, K. Cerney, J.Y. Hokama, G. Lai, R.F. Gonzales, G. Davis-Gorman and J.G. Copeland. Cardiovascular and Thoracic Surgery. The University of Arizona. Tucson, Arizona 85718

The recovery from cardiac surgery and cardiopulmonary bypass can be complicated by an acute inflammatory response. Circulating blood through an extracorporeal circuit (ECC) contributes to this complication. A perfluorocarbon (PFC)-based blood substitute is under investigation for use because PFCs increase the oxygen carrying capacity of the diluted blood. Some PFCs may also attenuate the ECC-induced inflammatory response. Earlier, we reported that perflubron emulsion ([PFE], *Oxygent*TM, Alliance Pharmaceutical Corp.) reduced neutrophil (PMN) activation in an in-vivo rat model (1). However, the potential of PFE to reduce ECC-induced PMN activation has not been investigated. In this study, we utilized a small-scale ECC model to quantify PMN activation during circulation and to examine if PFE treatment attenuated PMN activation. ECC circuits were filled with a mixture of rat blood and Plasma-Lyte (Baxter) and the dilute blood was circulated for 120 min. Two groups were studied, an untreated group containing blood plus Plasma-Lyte and a treated group in which some of the Plasma-Lyte was substituted with PFE (4.5g PFC/100 ml). Hematology and measures of whole blood, PMN activation were made from blood samples taken periodically during circulation. We found, for the untreated group, a decrease in the number of circulating PMNs and an increase in PMN activation with time. PMN activation was demonstrated as a progressive increase in the expression of the PMN adhesion protein, CD11b ($P < 0.05$). After 120 min of ECC, the PMNs remained capable of a significant response to a second inflammatory stimulus, but PFE treatment significantly attenuated the fMLP-induced increase in PMN oxygen free radical production (ROS) ($P < 0.05$). These results suggest that PFE may have dual utility in cardiac surgery, to increase oxygen delivery and to serve as an anti-inflammatory agent.

(1) Wilson et al. J.Extracorp.Technol. 29:123-131, 1997.

II-9. A 14-DAY INTRAVENOUS INFUSION TOXICOLOGY STUDY OF HEMOLINK™ (HLK) IN SPRAGUE-DAWLEY RATS

C. Moore, C.M. Woods and G.P. Biro. Hemosol Inc. Toronto, Canada

Adult rats (n=15/sex) received daily intravenous topline infusions of Ringer's Lactate (the excipient in HLK), or Pentaspan (oncotic control) at 30 ml/kg/day, or HLK at 5, 15 or 30 ml/kg/day (low-, mid-, high-dose) on 14 consecutive days (cumulative exposure of 60, 210 and 420 ml/kg HLK or about 1, 3.5 and 7 times the blood volume). Animals were sacrificed at one (n=10) or 14 days (Recovery; n=5) after the last dose. Clinical observations were recorded daily throughout the study. A full panel of hematology, clinical chemistry and urinalysis tests was performed at sacrifice, followed by necropsy and histopathological evaluation. Iron content of organs was evaluated by Perl's stain and by atomic absorption. There were no deaths and only limited clinical and toxicological findings, despite the prolonged exposure to high levels of circulating HLK. Feed consumption, growth rate and activity were decreased, and brown discoloration of the skin was observed in the mid- and high-dose HLK groups, but these were reversed at Recovery. At the end of dosing, bilirubin and BUN (but not creatinine) were elevated reflecting heme and protein metabolism, with pigments in urine. Liver weight increased in the mid- and high dose HLK and in the Pentaspan groups. At Recovery, organ weights were at or near control values. Histopathology revealed foamy histiocytes with pigment in several tissues and parenchymal cell alterations in sternal bone marrow, stomach and thymus. Parenchymal, but not histiocytic and pigmentary, changes were completely reversed at Recovery. In the High-dose HLK rats, iron content at Recovery was increased in all organs assayed: liver 7 times; heart: 1.7 times; kidney: 6.4 times; spleen: 3.3 times; lung: 2.3 times levels in Ringer's lactate control rats.

In conclusion, the effects observed were not degenerative and generally indicative of metabolism of the hemoglobin and iron accumulation and parenchymal changes were fully reversible. Therefore, the no-toxic effect level in unbled rats, following 14-days of recovery, was 30ml/kg/day, or a cumulative dose >20 times the exposure of a human to a dose of 1000 ml.

II-10. PRECLINICAL AND CLINICAL EXPERIENCE WITH A NOVEL HEMOGLOBIN ADENOSINE - GLUTATHIONE BASED RED CELL SUBSTITUTE

M. Feola¹, J. Simoni¹, and R. Angelillo². ¹Department of Surgery, Texas Tech University Health Sciences Center, School of Medicine, Amarillo, TX 79106 and Lubbock, TX 79430, and ²University of Naples' II Polyclinic, Naples, Italy

A novel red cell substitute developed at Texas Tech, consisting of bovine hemoglobin (Hb) cross-linked intramolecularly with *o*-ATP and intermolecularly with *o*-adenosine and conjugated with reduced glutathione (GSH), was subjected to preclinical and clinical testing. Preclinical testing included research done at Texas Tech and the IND study conducted at the Research Toxicology Centre S.p.A. (Rome, Italy). The research based on *in vivo* animal study and *in vitro* testing, using various human cell lines, was focused on the product's: -toxicity; -vasoactivity; -immunological, oxidative and inflammatory reactions; and -therapeutic potential. The IND study included: -unscheduled DNA synthesis in primary rat hepatocytes; -*in vitro* mammalian bone marrow cytogenetic test; -chromosome aberration in human lymphocytes *in vitro*; -reverse mutation in *Salmonella typhimurium*; -gene mutation in the Chinese hamster V79 cells; -test for foreign protein in the guinea pig; -delayed dermal sensitization study in the guinea pig; -acute intravenous toxicity in the albino rats; -seven day intravenous toxicity study in the albino rats; -acute toxicity study in dogs; -seven day intravenous toxicity study in dogs; -virus inactivation test; and -stability test. From this preclinical testing more than 80 abstracts and papers have been presented and published and an official IND report has been issued. The results of these studies are favorable, indicating that this novel red cell substitute has vasodilatory activity and can reduce the vasoconstriction that follows hemorrhage, has erythropoietic activities, and produces no adverse nephrotoxic, neurotoxic, oxidative or inflammatory reactions. The human clinical trial was performed at the Institut de la Recherche en Sciences de la Sante', Centre de l'Anemie S. S. (Kinshasa, Zaire). This published study (Feola M., Simoni J., Angelillo R., et al.: *Surg Gynecol Obstet* 174:379-386,1992) indicated no toxic or allergic reactions and beneficial effects in all patients tested. Together, based on preclinical and clinical testing, it can be concluded that this novel chemical/pharmacologic modification method has allowed the preparation of a non-toxic solution, which promises to be an effective, second generation, Hb-based human red cell substitute.

P-4. RECENT PROGRESS OF ARTIFICIAL BLOOD PROJECT AND NOVEL PRODUCTS

E. Tsuchida. Department of Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555, Japan

Ministry of Health and Welfare, Japan has started in 1997 the Artificial Blood Project as a part of the Advanced Research on Medical Care, and it continues the second stage during 2000 - 2002. This project is composed of artificial red cells to carry O₂, artificial platelets to induce blood coagulation, and artificial immune system to prevent infection. Among the three, the artificial red cell will be the first to meet the production with GMP standard aiming at clinical trials. As a consequence of the clinical studies of acellular Hb outside of Japan that revealed side effects such as hypertension by NO trapping, the recent trend is to increase the molecular dimension of Hb by polymerization or polymer conjugation. Cellular Hb-vesicles (HbV, 200 nm) have been developed in which a Hb-CO solution (35%) pasteurized at 60 °C is covered with a lipid bilayer membrane. After converting Hb-CO to Hb-O₂ under photoirradiation, the resulting HbV shows an appropriate O₂ affinity which is regulated by a coencapsulated allosteric effector. Surface modification with PEG suppresses intervesicular aggregation thus preserves homogeneous dispersion state. Met-Hb formation is suppressed in an anaerobic condition so that HbV can be stored in a shelf at room temperature for over one year.

On the other hand, synthetic lipidheme can bind O₂ reversibly in a physiological condition. Lipidheme-phospholipid vesicles (200 nmφ) and albumin-heme (8 lipidhemes are incorporated in one r-human serum albumin) are promising. Dimerization of the albumin molecule reduces its colloid osmotic pressure (COP) to half, thus 10wt% dimerized albumin-heme solution shows the same COP with blood and serves as an O₂ infusion with a sufficient O₂ transporting ability. Preparing regulated plant and the sample are going underway.

P-5. MECHANISMS OF FLOW INDUCED NO PRODUCTION

John Frangos, Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093

During the past two decades it has become evident that hemodynamic shear stresses are potent stimulators of endothelial function. Responses to shear include the release of vasoactive factors such as nitric oxide, prostaglandin I₂, and endothelin-1, as well as other growth modulators. Controversies were created, however, as contradicting results were being reported. By applying defined flow profiles, we were able to determine that hemodynamic shear stress represents two distinct mechanical stimuli to the endothelium. These are the temporal gradients in flow (rate of change of flow) and flow itself. It was shown that these mechanical stimuli are sensed and transduced by separate mechanochemical signal transduction pathways. Temporal gradients in flow induce a transient burst in nitric oxide production, via a pathway that involves heterotrimeric G-proteins and increases in intracellular calcium. Flow itself, however, leads to sustained nitric oxide production. Surprisingly, signaling occurs through a novel pathway that is both G-protein- and calcium-independent. These results have led to the hypothesis that responses of the endothelium to hemodynamic shear stress can be characterized as the superposition of the responses to the temporal gradients of shear stress and shear stress itself. The physiological and pathological relevance of these results will be discussed.

III-1. PLATELET SUBSTITUTES: AN OVERVIEW

J. Levin. Univ. of California School of Medicine and VA Medical Center, San Francisco, CA 94121

The need for platelet substitutes is based not only on the chronic shortage of donors, but also results from the 5 day 'shelf-life' of currently available platelet preparations. In addition, the requirement that fresh platelets be maintained at room temperature increases the risk of bacterial growth and the subsequent production of bacteremia in recipients. Platelet transfusions can also be associated with febrile reactions and alloimmunization. Thus, a variety of platelet substitutes have been considered or are under development, including cellular fragments of platelets (e.g., frozen-thawed platelet membranes), lyophilized paraformaldehyde-fixed platelets, lyophilized trehalose-loaded platelets, thromboerythrocytes (red blood cells coupled with RGD-containing peptides), liposomes that contain platelet glycoproteins, and albumin microspheres coated with fibrinogen. Because of the wide range of the hemostatic functions of fresh platelets, it is likely that most, if not all, platelet substitutes will support only some of the physiological functions of normal platelets. Therefore, platelet substitutes may have to be used selectively, depending upon the clinical need and the type of hemostatic deficiency that requires correction. The level of recovery and persistence in the circulation are important criteria for the efficacy of normal transfused platelets, for which there is a correlation between presence in the circulation and hemostatic effectiveness. However, for platelet substitutes there may be little correlation between retention in the circulation and hemostatic effect. Furthermore, it may be impossible to detect some types of substitutes in the circulation. No single in vitro test has been demonstrated to be a direct surrogate for platelet efficacy. Therefore, determination of the efficacy of platelet substitutes will be difficult, although multiple in vitro test systems and animal models are available. Ultimately, both safety and efficacy will have to be demonstrated in vivo, probably initially in patients who are not bleeding. However, currently there are no clinical tests adequate for determination of the efficacy of platelets or platelet substitutes. Determination of efficacy was considered at a recent workshop sponsored by the FDA, the NHLBI(NIH), and WRAIR (J.G. Vostal et al., Transfusion 40:742-750, 2000),

III-2. ADHESIVE PROPERTIES OF LIPOSOMES CARRYING RECOMBINANT GPIaIIa AND/OR GPIb α UNDER FLOW CONDITIONS

T. Nishiya¹, M. Suematsu² and Y. Ikeda¹. Department of ¹Internal Medicine, and ²Biochemistry, Keio University, Tokyo

Platelets have diverse functions, and it will be a formidable task to develop platelet substitutes that have an entire set of platelet functions. The simplest type of artificial platelets might be particles carrying platelet membrane proteins and/or their ligands involved in platelet adhesion and aggregation. Based on this idea, we prepared liposomes with recombinant GPIaIIa and/or GPIb α (rGPIaIIa-liposomes, rGPIb α -liposomes, and rGPIaIIa-Ib α -liposomes), and evaluated their adhesive properties under flow conditions. rGPIaIIa-liposomes efficiently adhered to the collagen surface at low shear rates, but not at high shear rates¹. rGPIb α -liposomes reversibly interacted with the vWf surface under flow conditions, depending on the shear rate and the densities of receptor and matrix². rGPIaIIa-Ib α -liposomes efficiently adhered to the collagen surface even at high shear rates¹, indicating that two distinct receptor-ligand pairs, rGPIaIIa/collagen and rGPIb α /vWf, complement each other, and synergistically provide the needed functional integration required for adhesion under unfavorable shear forces. Liposomes in mesenteric microvessels can be visualized with an intravital microscope assisted by ultrahigh speed intensified video imaging system. rGPIb α -liposomes exhibited a transient adhesion to the endothelium in the millisecond range, and this event was attenuated by monoclonal antibodies against GPIb α (GUR83-35) or vWf (AJvW-2), similar to platelets.

T. Nishiya *et al.*, Blood, 94 (Suppl. 1) 443a, 1999.

T. Nishiya *et al.*, BBRC, 270: 755-760, 2000.

III-3. INTRACELLULAR STIMULUS-RESPONSE COUPLING AND POSITIVE FEEDBACK AMPLIFICATION OF HEMOSTATIC FUNCTIONS WITH REHYDRATED, LYOPHILIZED PLATELETS

T.H. Fischer, PhD¹, E.P. Merricks¹, R.A. Raymer¹, A.P. Bode, PhD², and M.S. Read, PhD¹.

¹Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC and ²Department of Pathology, East Carolina University, Greenville, NC

We have developed methods, based on mild chemical cross-linking and lyophilization, for preserving platelets for long-term storage. The goal of these studies was to determine if rehydrated, lyophilized platelets were capable of activation-dependent intracellular signaling, and if this stimulus-response coupling that was capable of driving platelet activation reactions for positive feedback amplification of hemostatic reactions. We first examined intracellular signaling pathways for stimulus-response coupling. Next we investigated the extent to which RL platelets undergo activation responses that mediate positive feedback in thrombus formation. Exposure of RL platelets to thrombin resulted in enhanced phosphorylation of several intracellular proteins, including 18 kDa and 42 kDa kinase substrates which were shown to be the myosin light chain (substrate for myosin light chain kinase) and pleckstrin (substrate for protein kinase C). Cross-linking and lyophilization depleted the platelets of free cytoplasmic ATP, but had less of an effect on protein-bound nucleotides; the protein-bound nucleotides were capable of supporting energy-dependent intracellular kinase activities and cytoskeletal functions. These results indicate that RL platelets retain some of the activation-dependent intracellular functions of fresh platelets. We performed two experiments that showed that RL platelets can drive positive-feedback amplification in hemostasis. First, we demonstrated that RL platelets retain sufficient energy-dependent cytoskeletal functions to undergo an activation-dependent secretion response. Secondly, the thrombogenicity of RL platelets, which is in part mediated by activation-dependent surface exposure of phosphatidylserine, increases in an activation-dependent manner. These results help explain how RL platelets provide an immediate and persistence hemostatic effect in cardiopulmonary bypass-induced platelet enemia.

III-4. THE SIGNIFICANCE OF TEMPERATURE FOR PLATELET STORAGE AND THE DEVELOPMENT OF PLATELET SUBSTITUTES

F. Tablin¹, N.J. Walker¹, W. Wolkers², and J. Crowe². ¹School of Veterinary Medicine and ²Division of Biological Sciences; University of California – Davis, Davis, CA 95616

The phenomenon of cold-induced platelet activation has been recognized since 1954 (Zucker and Borelli Blood 9:602-608). It has precluded cold storage of platelets, as cells incubated at 4°C for > 24 hrs undergo irreversible shape change, the production of microvesicles and the fusion of alpha and dense granules and their eventual secretion. All of these factors have led to the current practice of blood banking platelets at 22°C for no more than 5 days. Our laboratories have shown that cold-induced platelet activation is closely correlated with the passage of intact platelets through their phospholipid phase transition (Tablin et al. J. Cell Physiol. 168:305-313, 1996). Additionally, we have shown that there is a rise in intracellular calcium that correlates not only with the intact platelet membrane phase transition, but also with the phase transition of the dense tubular system (the calcium storage organelle) (Oliver et al. Biophysica Biochemica Acta 1416:349-360, 1999) Our data correlate with that of Winokur and Hartwig (Blood 85:1494-1503, 1995) demonstrating a net increase in filamentous actin during cold-induced activation. We have recently developed a method to produce stable freeze-dried human platelets by freeze-drying them with trehalose, a sugar found at high concentrations in organisms that naturally survive drying. Trehalose is rapidly endocytosed by human platelets at 37°C, with loading efficiencies of 50% or greater. Trehalose-loaded platelets were successfully freeze-dried with excellent recovery of platelets (85%). Aggregometry of rehydrated freeze-dried platelets demonstrated they were responsive to thrombin, collagen, ADP and ristocetin in an almost identical manner to fresh platelet controls. Analysis of rehydrated freeze-dried platelets by Fourier transform infrared spectroscopy demonstrated that there was normal protein secondary structure and normal membrane phase transitions. This work is supported by a grant from DARPA (Navy) N66001-00-C-8048.

III-5. MECHANISM OF ACTION OF INFUSIBLE PLATELET MEMBRANES

P.M. Hilarius¹, G. Escolar², M. Gendreau³ and A.J. Verhoeven¹. ¹CLB Sanquin, Amsterdam; ²Academic Hospital, Barcelona; ³Cypress Bioscience Inc., La Jolla, CA

Aim of the study. When platelets are subjected to repeated freeze-thawing and extensive washing, the resulting preparation (Infusible Platelet Membranes, IPM) is able to stop bleeding in thrombocytopenic rabbits and humans. Hence, IPM can be considered as potential platelet substitute. In this study, the mechanisms involved in the hemostatic function of IPM were investigated.

Materials and methods. IPM were prepared from outdated platelets according to published procedures, except that the preparations were not lyophilized and that, in some preparations, heat treatment (for pathogen inactivation) was omitted. To check for integrity of platelet antigens, antibodies specific for glycoprotein (GP)1b and GPIV, were used in different assays. Biological activity was assessed in thrombocytopenic rabbits by measurement of ear bleeding times. Procoagulant activity was measured in whole plasma incubated with Russell's Viper Venom protease and Ca²⁺ or in a flow system with thrombocytopenic blood on a subendothelial matrix.

Results. Both GP1b and GPIV were found to be destroyed by the heat treatment, that is part of the published production procedure of IPM. Previous work had already shown the absence of GPIIb/IIIa. Despite the absence of proteins relevant for platelet function, the IPM preparations were active in thrombocytopenic rabbits, indicating another supporting effect. In two different systems, procoagulant activity could easily be demonstrated. In agreement with this notion, IPM preparations stained highly positive with Annexin V-FITC. However, procoagulant activities did not correlate exactly with hemostatic effects in the rabbit model.

Conclusion. Our results indicate that for the hemostatic function of IPM not so much the protein, but rather the lipid content is important.

P-6. OXIDATIVE MECHANISMS OF HEMOGLOBIN-BASED BLOOD SUBSTITUTES

A.I. Alayash, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

Cell-free hemoglobins, chemically altered or genetically expressed in microbial host systems, have been developed as oxygen-carrying therapeutics. Site-directed modifications are introduced and serve to stabilize the protein molecules in a tetrameric and/or a polymeric functional form. Direct cytotoxic effects associated with cell-free hemoglobin (Hb) have been ascribed to redox reactions (involving either 1 or 2 electron steps) between the heme group and peroxides. These interactions are the basis of the pseudoperoxidase activity of Hb and can be cytotoxic when reactive species are formed at relatively high concentrations during inflammation and typically lead to cell death. Peroxides relevant to biological systems include hydrogen peroxide (H_2O_2), lipid hydroperoxides (LOOH), and peroxynitrite (ONOO^-). Reactions between Hb and peroxides form the ferryl oxidation state of the protein, analogous to compounds I and II formed in the catalytic cycle of many peroxidase enzymes. This higher oxidation state of the protein is a potent oxidant capable of promoting oxidative damage to most classes of biological molecules. It has become increasingly evident that Hb redox reactions or their byproducts play a critical role in the pathophysiology of oxidant-induced injury in some disease states. Further complications are thought to arise from the reactions of Hb and biological peroxides and their impact on modulation of cell signaling pathways regulated by these reactive species.

IV-1. SELENIUM REDUCES HEMOGLOBIN-INDUCED EPITHELIAL DAMAGE TO INTESTINAL MUCOSA

A.L. Baldwin and E.B. Wiley. Department of Physiology, College of Medicine, University of Arizona, Tucson, AZ 85724-5051

Modified hemoglobins are being considered as possible "blood substitutes". Previously, we showed that injection of PEG-conjugated hemoglobin into the circulation of rats caused epithelial sloughing (Baldwin et al, 1998). The present study was performed to determine whether diaspirin cross-linked hemoglobin (DBBF-Hb, U.S. Army, gift from Dr. A. Alayash, FDA) produces epithelial damage and, if so, whether it is reduced in rats receiving supplemental selenium (Se), daily, for 3 weeks prior to injection. Anesthetized Sprague-Dawley rats, half of which received 2×10^{-6} g/ml Se in their drinking water, were injected, arterially, with a 5 ml bolus of 10 mg/ml DBBF-Hb in Hepes buffered saline (HBS) with 2% bovine serum albumin (BSA). Control animals received HBS-BSA alone, (5 animals per group). After 30 min., the intestine was perfusion-fixed for light and electron microscopy. Eighty villi were examined per rat and assigned an epithelial integrity index (E.I.I.), ranging from 1 (intact) to 3 (some cell-cell and cell-basement membrane separation). In non-Se rats, epithelial integrity was significantly compromised by DBBF-Hb, compared to HBS-BSA (2.47 ± 0.57 (SD) vs 1.36 ± 0.49 , $p < 0.001$). In Se rats, neither injection with DBBF-Hb, nor with HBS-BSA, caused epithelial damage (1.03 ± 0.17 vs 1.07 ± 0.26). Sixty villi from each rat were examined to count mast cell degranulation (MCD) per villus. In non-Se rats, MCD was significantly greater after DBBF-Hb than after HBS-BSA injection (1.83 ± 1.42 vs 0.2 ± 0.4). Supplementary Se did not reduce this effect. In fact, MCD was significantly increased in both sets of rats compared to their non-Se counterparts: (3.27 ± 2.40 and 1.48 ± 1.70 for DBBF-Hb and HBS-BSA, respectively). Since many mast cell mediators cause cell damage, it appears that Se has an overwhelming protective effect on the mucosal epithelium.

A.L. Baldwin, L.M. Wilson and J.E. Valeski. Am. J. Physiol. 275:H615-H625, 1998.

IV-2. REDOX REACTIONS OF HEMOGLOBIN ALTERS THIOL LEVELS AND THE MODE OF ENDOTHELIAL CELL DEATH

F. D'Agnillo and A.I. Alayash. Center for Biologics Evaluation and Research, FDA, Bethesda, MD, 20892

Heme-mediated redox reactions are thought to contribute to organ dysfunction and/or tissue damage that occurs with the use of hemoglobin-based oxygen carriers. Endothelial cells are primary targets for injury and/or dysfunction resulting from the interaction of hemoproteins with reactive oxygen and/or nitrogen species. We investigated the pathways of cellular injury and death in normal and glutathione (GSH)-depleted bovine aortic endothelial cells (BAECs) subjected to bolus hydrogen peroxide (H_2O_2) or steady state levels of H_2O_2 produced by the glucose-glucose oxidase system. Diaspirin cross-linked hemoglobin (DBBF) was converted to its highly toxic, ferryl form (DBBF- Fe^{4+}) when incubated with bolus or enzymatically-generated H_2O_2 . The formation of DBBF- Fe^{4+} was correlated with a significant loss of intracellular GSH compared to H_2O_2 or DBBF alone. DBBF- Fe^{4+} formation and the loss of GSH were inhibited by catalase, but not by superoxide dismutase or desferoxamine. BAECs incubated with DBBF and steady state levels of H_2O_2 underwent severe cellular rounding, swelling, and detachment, and accumulated in the G2/M phase of cell cycle. Cell cycle arrest in the G2/M phase preceded the onset of death by apoptosis as revealed by increased phosphatidylserine externalization and DNA fragmentation measured by annexin V assay and sub G1/G0 events, respectively. The addition of catalase inhibited cell cycle arrest and apoptosis triggered by the combination of DBBF and H_2O_2 . Cell death and injury was more pronounced in cells with depleted GSH defenses induced by pretreatment with buthionine sulfoximine, an inhibitor of GSH synthesis. These findings may have important implications for the use of hemoglobin-based oxygen carriers in patients lacking antioxidant defenses due to co-existing pathologies, and may provide insights into designing prevention strategies.

IV-3. HEMOGLOBIN-DEPENDENT NEUROTOXICITY

R. F. Regan¹ and S. S. Panter². ¹Thomas Jefferson University, Philadelphia, PA 19107; ²University of California San Francisco and San Francisco Veterans Administration Hospital, San Francisco, CA 94121

Initial studies performed in co-cultures of neurons and astrocytes found that low-micromolar concentrations of hemoglobin killed the neurons. Pharmacological inhibitors were used to demonstrate that death could be partly attributed to oxidative injury. Recently, we have used a probe that detects oxygen radical generation, 2-dichlorofluorescein (DCF) diacetate, to quantify free radical production following exposure to hemoglobin. This probe freely enters cells and is fluorescent when oxidized by free radicals. At 4 and 6 hours after hemoglobin exposure, fluorescence was increased 30-fold and 115-fold, respectively. Neurons from animals lacking the gene for heme oxygenase isoenzyme-2 (HO-2 knockouts) were found to be significantly less sensitive to hemoglobin-dependent cell death. Neurons loaded with DCF manifested significantly reduced radical production. We have also recently performed *in vivo* experiments in which hemoglobin was injected intravascularly following hyperosmotic disruption of the blood-brain-barrier. Brain sections were subjected to immunohistochemistry for a neuron-specific protein (Neu-N) and heat shock protein 70 (HSP70), a protein that is synthesized in injured cells. Significant areas of neuronal loss in cortex were detected by Neu-N, and diffuse as well as focal areas of HSP70 expression were observed in both cortex and hippocampus. In another *in vivo* model, stroma-free hemoglobin was injected directly into gerbil hippocampus, and the brains were examined for injury 24 hours later. Large infarcts were observed in the hippocampi of animals that received hemoglobin but not in controls that received human serum albumin. These data led us to postulate that hemoglobin might have deleterious effects in the human central nervous system.

IV-4. EFFECTS OF DEX-BTC-Hb AND $\alpha\alpha$ -Hb ON HUMAN AORTIC ENDOTHELIAL CELL FUNCTIONS *IN VITRO*

Toussaint, M.¹, Devaux, Y.², Caron, A.¹, Muller, S.³, Stoltz, J.F.³, Longrois, D.², Menu, P.¹, and Vigneron, C.¹. ¹Department of Hematology-Physiology, ² Department of Experimental Surgery, ³Department of Angiohematology, University Henri Poincaré Nancy 1, 54001 Nancy, France

Cell-free hemoglobin has been demonstrated to penetrate inside endothelial cells and to interact with endothelium-derived factors (NO, endothelin-1), which results in the vasoactive effects of HBOCs. We therefore hypothesized that these solutions should modify endothelial cell functions by altering the expression of some genes, in particular the constitutive isoform of endothelial NO synthase (NOS3) and/or by activating cells. After incubation of cultured human aortic endothelial cells (HAEC) with Dex-BTC-Hb or with $\alpha\alpha$ -Hb (16 g/L, clinically relevant concentration) for 3 hours, NOS3 ARNm and protein were extracted and their expression were assessed by semi-quantitative RT-PCR and Western blot respectively. The expression and localization of the adhesion molecule ICAM-1 which reflects the activation state of the cells were detected by fluorescence microscopy. Neither Dex-BTC-Hb nor $\alpha\alpha$ -Hb induced any change in NOS3 ARNm and protein levels immediately or 24 hours after incubation, suggesting that these HBOCs do not influence the expression of this gene. In addition, trapping of NO by cell-free hemoglobin may not result in a variation of NOS3 expression. The expression and the localization of ICAM-1 on the cell membrane were modified by incubation of cultured HAEC with the 2 HBOCs tested for 3 hours, suggesting an activation of the endothelial cells by these HBOCs. This activation may be due to oxidative stress generated by hemoglobin oxidation. Dex-BTC-Hb and $\alpha\alpha$ -Hb at 16 g/L did not alter NOS3 gene expression but appeared to activate human endothelial cells *in vitro* which may have impact *in vivo* on the endothelial cells/leukocytes interactions.

P-7. MICROVASCULAR, MECHANICAL & CELLULAR BASIS FOR EFFECTIVE BLOOD SUBSTITUTES

M. Intaglietta. Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093

Oxygen carrying plasma expanders based on molecular hemoglobin solutions are in clinical trials and may soon be available. However, they are designed following concepts representative of the physiological know how of past decades, which focused on reproducing the oxygen transport properties of blood and the assumption that it is generally beneficial to lower blood viscosity. A further hindrance in producing an effective alternative to blood was the incomplete understanding of oxygen regulation and transport in the microcirculation. Recent technology for measuring oxygen show that in the microcirculation oxygen is delivered primarily by arterioles, and that alteration of the fluid properties of blood can selectively direct most of the oxygen to the arterioles per se, to the detriment of tissue oxygenation. The inherent low viscosity of most hemoglobin solutions alters the distribution of shear stress changing the production of NO, prostacyclin and endothelin leading to vasoconstriction and apoptosis, which lower functional capillary density. Detailed oxygen measurements in the microcirculation during hemorrhage show that survival is firstly critical dependant on maintenance of functional capillary density, and secondly on tissue oxygen. Taken as a whole these findings lead to the design of a new generation of materials where the effectiveness of oxygen transport transport in the microcirculation by hemoglobin molecule is insured by modifications that result in large molecular radii, yielding high viscosity solutions. Oxygen delivery is targeted to regions of low oxygen by using high affinity, and oncotic pressures are high to induce fluid volume shifts which lower infusion volumes, and cause the rapid recovery of systemic and capillary pressure in hemorrhage. These new materials, specifically designed to insure the maintenance of microvascular function through the release of endothelial derived factors that are present when the tissue is perfused with normal blood, insure the maintenance of capillary flow, and may be more efficacious than blood when used as therapeutic agents.

P-8. OXYGEN AND BLOOD FLOW REGULATION: THE SEARCH FOR THE MISSING LINK

P.C. Johnson, A. Tsai and M. Intaglietta. Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093-0412

The single most important function of the circulation on a moment-by-moment basis is the delivery of oxygen to the tissue. Blood flow to many organs appears to be regulated such that the parenchymal cells receive an adequate supply of oxygen while preventing an over-abundance. Despite over a century of study the mechanisms involved are still not clear. One hypothesis suggests that there are normally tissue areas on the borderline of hypoxia and these areas produce vasodilator agents such as adenosine, H^+ ion, and inorganic phosphate. Our studies in resting skeletal muscle do not indicate that there are normally tissue areas where tissue pO_2 is below critical levels. However, this can occur when blood flow is reduced by about 50%. During increased metabolic demand such as in contracting muscle the above-mentioned metabolic vasodilators and others such as potassium and increased osmolarity may become important. Recent studies have provided evidence that in certain organs, vasodilator autacoids, specifically prostaglandins and nitric oxide, are released in increased amounts from the endothelium as arteriolar pO_2 falls. In addition deoxygenation of hemoglobin in transit through the arteriolar network causes release of NO and ATP from the red cell. There is also evidence that the vasoconstrictor autacoids lipoxxygenase and a metabolite of cytochrome P450, 20-HETE are released in increased quantities as pO_2 rises. Moreover, it appears that the arterioles are not the only sensors, the capillary network may be involved in sensing and transmitting information on metabolic status as well. These findings suggest that autacoids may be principally important for regulation in resting muscle and maintain tissue oxygen levels within narrow limits through oxygen-dependent release of vasodilator and vasoconstrictor agents. Vasodilator products of energy metabolism may become more important when oxygen delivery decreases or consumption rises.

V-1. COMPARATIVE EFFECTS OF CROSS-LINKED, CONJUGATED AND POLYMERIZED HEMOGLOBINS ON HEMODYNAMICS AND BLOOD VISCOSITY AFTER MODERATE HEMODILUTION IN ANESTHETIZED RABBITS

A. Caron¹, P. Menu¹, A.I. Alayash², P. Labrude¹, and C. Vigneron¹. ¹University Henri Poincaré-Nancy 1, 54001 Nancy, France, ²Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892

Variously modified hemoglobins with diverse physico-chemical properties have been shown to produce variable hemodynamic responses. We compared the systemic and renal hemodynamic effects of bis(3,5-dibromosalicyl)fumarate crosslinked ($\alpha\alpha$ -Hb), dextran-benzene-tetracarboxylate-conjugated (Hb-Dex-BTC) and *o*-raffinose-polymerized (*o*-raffinose-Hb) hemoglobins perfused in anesthetized rabbits undergoing moderate hemodilution (30% hematocrit). Two plasma volume expanders, albumin 5% and hydroxyethyl starch 6%, were used as reference solutions. In addition, vascular hindrance (resistance/blood viscosity measured at 128.5 s⁻¹) was calculated to determine whether a moderate decrease in the viscosity of blood mixed with HBOCs may impair vasoconstriction following infusion of cell-free hemoglobin. No changes were observed in renal blood flow following hemodilution with either reference or hemoglobin solutions. Increase in both blood pressure and vascular resistance was observed with Hb-Dex-BTC and $\alpha\alpha$ -Hb (for 180 min) and to a lesser extent with *o*-raffinose-Hb (for 120 min). Furthermore, Hb-Dex-BTC (viscosity: 2.15 cP) and *o*-raffinose-Hb (viscosity: 1.26 cP) induced comparable increases in vascular hindrance (from 0.091 to 0.159 cm⁻¹ and from 0.092 to 0.162 cm⁻¹, respectively) but far less than that produced by $\alpha\alpha$ -Hb (viscosity: 0.99 cP; from 0.092 to 0.200 cm⁻¹). These results indicate that maintaining post-hemodilution blood viscosity by infusing solutions with high viscosity may limit vasoconstriction due to autoregulation mechanisms and to pharmacological properties of cell-free hemoglobin.

V-2. THE EFFECTS OF HEMOGLOBIN GLUTAMER-200 [BOVINE] ON THE MICROCIRCULATION IN A CANINE HYPOVOLEMIA MODEL: AN INTRAVITAL MICROSCOPY STUDY

A.T.W. Cheung¹, J.S. Jahr¹, B. Driessen², P.L. Duong¹, M.S. Chan¹, and R.A. Gunther¹.

¹University of California, Davis School of Medicine, Davis, California, 95616 and ²University of Pennsylvania School of Veterinary Medicine, Kennett Square, PA 19348

Hypovolemic dogs were resuscitated with Hemoglobin Glutamer-200 [Bovine] (HB-200) (n=4), using autologous shed blood resuscitation (n=4) as control. Systemic, hemodynamic and oxygenation measurements were made and microvascular characteristics of the bulbar conjunctiva were non-invasively and simultaneously quantified using computer-assisted intravital microscopy. All dogs (n=8) showed significant *post-hemorrhagic* changes in microvascular characteristics (venular diameter, vessel density, avascularity and blood flow velocity). Induced hemorrhage ($\leq 40\%$ blood loss) reduced mean arterial pressure to 50mm Hg and was accompanied by significant impairments in systemic functions and oxygenation. Shed blood resuscitation returned all measured systemic functions and oxygenation parameters to *pre-hemorrhagic* values and induced significant improvements ($P < 0.01$) in microvascular characteristics. In contrast, HB-200 resuscitation restored most systemic functions to *pre-hemorrhagic* values, but failed to restore hematocrit, total hemoglobin, cardiac output, oxygen delivery index and systemic venous resistance to *baseline* values. HB-200 resuscitation induced significant microvascular improvements ($P < 0.01$) in all four dogs. Microvascular improvements observed immediately (*resuscitation 1*) after HB-200 and autologous blood resuscitations did not change significantly through the 3-hr post-resuscitation observational period (*resuscitation 2*). In addition, HB-200 resuscitation in hypovolemic dogs with a 40% blood volume reduction did not result in extreme hemodilution or cause any fatal outcome. An animal model and relevant intravital technologies are now available to study microvascular, systemic, hemodynamic and oxygenation changes simultaneously in future hypovolemic studies.

V-3. SYSTEMIC AND MICROCIRCULATORY EFFECTS OF AUTOLOGOUS WHOLE BLOOD TRANSFUSION IN SEVERE 4-HOUR HEMORRHAGIC SHOCK

H. Kerger¹, K.F. Waschke¹, A.G. Tsai² and M. Intaglietta². ¹Department of Anesthesiology, University Hospital of Mannheim, Germany; ²Department of Bioengineering, University of California San Diego, La Jolla, USA

After approval by the animal care committee, efficacy of autologous blood transfusion in restoring macro- and microhemodynamics after a 4-h hemorrhagic shock (MAP=40 mmHg) was studied in 63 conscious Syrian golden hamsters. Subcutaneous microcirculation was visualized in a dorsal skinfold, while shock was induced by withdrawal of blood volume (50%) which was re-transfused after 4 h. Systemic parameters were mean arterial pressure, heart rate and base excess (BE), while in the microcirculation arteriolar and venular blood flow, vessel diameters and functional capillary density (FCD) were determined. Microvascular and interstitial pO₂ (pO_{2 int}) were measured by phosphorescence quenching. According to 24-h outcome, animals were grouped into survivors in good (SG) and poor conditions (SP), and non-survivors (NS). Hemorrhagic shock caused severe metabolic acidosis, combined with a decrease in FCD, blood flow and pO_{2 int} [1.8 (SG), 1.3 (SP) and 0.9 (NS) vs. 23.0 mmHg (control)]. Transfusion restored macrohemodynamics in all animals, while metabolic acidosis and a 40-60% decrease of pO_{2 int} persisted. NS (44.4%) exhibited more severe systemic and microcirculatory alterations both in shock and after resuscitation. SP showed significant metabolic acidosis and reduction of FCD and pO_{2 int} (>50%) after 24 h, while SG (31.8%) showed only slight impairment of these parameters (15-30%). Overall, blood transfusion caused only a partial restoration of tissue perfusion and oxygenation. Differences in outcome (SG, SP, NS) were primarily related to BE, FCD and pO_{2 int}.

V-4. DOES THIOL PLAY A ROLE IN HEMOGLOBIN MEDIATED VASOACTIVITY IN THE ISOLATED RAT THORACIC AORTA?

H.W. Kim and A.G. Greenburg. Brown University and The Miriam Hospital, Providence, Rhode Island

In addition to heme-irons, reactive thiol groups (eg., $\beta 93$ Cysteine) of hemoglobin (Hb) also have been shown to react with nitric oxide (NO) producing S-nitroso compounds. What are relative contributions of these NO binding sites to the overall Hb vasoactivity? We investigated this question using a functional bioassay. Thoracic aorta rings with intact endothelium were prepared from male SD rats, suspended in oxygenated Krebs buffer, preconditioned with norepinephrine and/or acetylcholine, and isometric tension responses to test Hbs evaluated. Based on site specific reaction chemistries, tests Hbs were prepared by reacting purified human Hb with potassium ferricyanide and/or N-ethyl maleimide (NEM; a cysteine specific blocking reagent). Test Hbs prepared were HbCN, a Hb with blocked heme-iron sites, NEM-Hb, a Hb with masked cysteine residues, and NEM-HbCN, a Hb with both heme and cysteine sites blocked. Vascular ring tension responses to test Hbs were compared with that of unmodified human oxyHb (HbO₂; control). Typically treatment with control Hb elicited a significant contraction; 0.4 μ M Hb caused 58.1 \pm 19.7% (Mean \pm SD) increase in vessel ring tension over the pretreatment value ($P < 0.01$, N=8, Student's t-test). NEM-Hb at 0.4 μ M also caused a significant contraction (50.7 \pm 26.8%, $P < 0.01$, N=8) indicating that masking cysteines does not significantly attenuate Hb vasoactivity. In contrast, HbCN did not produce notable contraction (-3.7 \pm 11.8%, N=7, $P > 0.05$) suggesting importance of reactive heme sites in Hb vasoactivity. Masking cysteine residues as well (NEM-HbCN) had no further effect. When ferrous Hbs, with or without masked cysteines, were preliganded with NO (HbNO and NEM-HbNO, respectively), they did not elicit any significant contraction (-5.5 \pm 15.9%, N=7, $P > 0.05$ and 3.2 \pm 3.0%, N=3, $P > 0.05$, respectively). Additionally, ferrous sperm whale myoglobin (Mb), which has no cysteine residue, elicited a significant contraction (55.1 \pm 41.8%, N=10, $P < 0.01$) while metMb did not (-0.8 \pm 7.6%, N=6, $P > 0.05$). Finally, equine heart cytochrome C, a heme protein with both the heme iron and cysteine binding sites naturally blocked, did not elicit notable contraction at all (-6.8 \pm 13.5%, N=10, $P > 0.05$). These results indicate that reactive cysteine residues do not appear to play a primary role in Hb induced vasoactivity in the isolated rat thoracic aorta.

V-5. HEMODILUTION WITH HEMOLINK™ DOES NOT ADVERSELY AFFECT LV HEMODYNAMICS OR BLOOD FLOW DISTRIBUTION IN ANESTHETIZED DOGS

J.G. Kingma, Jr.¹, N.D. Hamelin², D. Gendron², Y. Trudel², M. Bosa², R. Stewart², M.B. Fargey², and G.P. Biro³. ¹Laval University, QC, ²ITR Laboratories Canada Inc., Montreal, QC, ³Hemosol Inc., Toronto, ON, CANADA

Hemoglobin based oxygen-carrying solutions (HbOCs) as hemoglobin replacement therapeutics are being tested for clinical use. Some of these products may be associated with elevations in both systemic and pulmonary vascular resistances. Effects on the distribution of blood flow (BF) to major organs and regional resistances have not been extensively described. We tested two formulations of *o*-raffinose cross-linked human hemoglobin, Hemolink™ (frozen HLK-1 and refrigerated HLK-2) and compared them to the colloid in extensive clinical use, Pentaspan® (Pen). Cardiovascular measurements and arterial blood gases were determined along with distribution of BF (radiolabeled microspheres) to the major organs in Beagle dogs (n=5 per group). After baseline measurements either HLK-1, or HLK-2, or Pen was exchange transfused (hematocrit reduced to approximately 25%). Measurements were repeated at 30, 60, 120 and 180 min post-exchange. In all groups cardiac output increased, but relative to Pen (marked but shorter lived increase) HLK-exchanged dogs were better able to maintain cardiac output. Systemic vascular resistance decreased after exchange but was reduced less with HLK. Pulmonary arterial pressure was higher in all groups; rate-pressure product (LV systolic pressure X heart rate) was also higher after HLK compared to Pen. Myocardial BF increased in all groups but was more sustained with HLK; endo-/epicardial flow ratios were maintained. In all groups, BF to brain (white and grey matter) increased at 30 min but declined thereafter to near baseline values. No significant change in renal cortical BF was noted. In conclusion, partial exchange transfusion with Hemolink™, similar to that used in acute normovolemic hemodilution, does not significantly alter cardiac hemodynamics and maintains distribution of BF to all major organs. Hemosol Inc., Canada provided Hemolink™ and funded this study.

V-6. EFFECTS OF *OXYGENT*TM ON BLOOD OXYGENATION IN CAPILLARIES FOLLOWING ISOVOLEMIC HEMODILUTION

Maeng, P.S. and Pittman, R.N. Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298

The current study examined the effects of isovolemic hemodilution with *Oxygent* (60% w/v perfluorochemical emulsion) on oxygen transport in capillaries of the hamster retractor muscle. We used severe isovolemic hemodilution (systemic Hct decreased from 52% to 16%) to stress the oxygen transport system of two groups of hamsters. In one group (N=12) Hespan, a non-oxygen carrying plasma expander, was used as the hemodiluent; in the other group (N=10), *Oxygent* (Alliance Pharmaceutical Corp., San Diego) was used (2.7 g/kg final concentration). The retractor muscle was prepared for intravital microscopic observations in anesthetized hamsters who breathed room air. The partial pressure of oxygen, PO₂, was measured at the arteriolar and venular ends of capillaries using phosphorescence quenching microscopy. Arteriolar capillary PO₂ decreased from 49 to 28 mmHg in the Hespan group, and decreased from 51 to 34 mmHg in the *Oxygent* group. Venular capillary PO₂ decreased from 37 to 21 mmHg in the Hespan group, and decreased from 41 to 27 mmHg in the *Oxygent* group. PO₂ values at both the arteriolar and venular ends of capillaries in the *Oxygent* group were 6 mmHg higher than the corresponding values for the Hespan group. Although this difference was substantial, in terms of being in the steep part of the oxygen dissociation curve, it was not statistically significant, due primarily to the large intrinsic heterogeneity of the PO₂ values in the hemodiluted state for the Hespan group (coefficient of variation, CV = 51%) compared with the more homogeneous oxygenation (CV = 31%) associated with the *Oxygent* group. It is noted that these results were obtained under conditions where the animals were breathing room air, rather than a mixture enriched with oxygen, as is the more usual condition for tests with perfluorocarbon emulsions. One would expect that future tests with elevated inspired PO₂ would yield substantially higher oxygenation of capillary blood, given the higher oxygen solubility provided by *Oxygent* compared with plasma or a plasma expander.

V-7. MOLECULAR DIMENSIONS OF Hb-BASED O₂ CARRIERS DETERMINE CONSTRICTION OF RESISTANCE ARTERIES AND HYPERTENSION*

H. Sakai¹, H. Hara¹, M. Yuasa¹, A.G. Tsai², S. Takeoka¹, E. Tsuchida¹, and M. Intaglietta².

¹Department of Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555, Japan, and

²Department of Bioengineering, University of California, San Diego, La Jolla, 92093-0412

The effect of molecular size of Hb-based O₂ carriers on the diameter and blood flow of resistance arteries (A₀, diameter, 158 ± 21 μm) and mean arterial blood pressure (MAP) were studied in the conscious hamster dorsal skinfold model. Intra-molecularly crosslinked Hb (XLHb), PEG-conjugated pyridoxalated Hb (PEG-PLP-Hb), hydroxyethylstarch-conjugated XLHb (HES-XLHb), glutaraldehyde-polymerized XLHb (Poly-XLHb) and PEG-conjugated Hb-vesicles (PEG-HbV: cellular type) were synthesized. Their molecular diameters were 7, 22, 68 and 224 nm, respectively. The top load infusion of 7 ml/kg of XLHb (5 g/dl) caused the immediate increase of MAP, which was 34 ± 13 mmHg higher 3 hrs after infusion. There was a simultaneous decrease in diameter of A₀ vessels (79 ± 8% of basal value) which caused blood flow to decrease throughout the microvascular network. The diameter of smaller arterioles did not change significantly. Infusion of O₂ carriers of greater molecular size resulted in lesser vasoconstriction and hypertension, PEG-HbV showing the smallest changes. Infusion of human serum albumin was used as control and produced no microvascular or systemic effects. Constriction of resistance arteries was found to be correlated to the level of hypertension, and the responses proportional to the molecular dimensions of Hb-based O₂ carriers. The underlying mechanism is not evident from these experiments, however, since the results correlate with molecular size it is likely that the effects are related to the diffusion properties of the different Hb molecules.

* H. Sakai, H. Hara, M. Yuasa, A.G. Tsai, S. Takeoka, E. Tsuchida, and M. Intaglietta. *Am. J. Physiol. Heart Circ. Physiol.* 2000;279, 908-915.

P-9. PRESENT STATUS OF NEW-GENERATION HEMOGLOBIN PRODUCTS

T.-M.-S. Chang. Director, Artificial Cells & Organs Research Centre, Professor of Physiology, Medicine & Biomedical Engineering, Faculty of Medicine, McGill University, Montreal, P.Q. Canada H3G 1Y6

Ongoing Phase III clinical trials have demonstrated the safety and efficacy of a number of first generation blood substitutes especially in in perioperative uses. With the coming of first generation blood substitutes for clinical uses, studies are being carried out into new generations of hemoglobin products for other clinical situations. One area of special interest is in ischemia-reperfusion that could be present in prolonged severe hemorrhagic shock, stroke, myocardial infarction, organ transplantation and other conditions. Studies include our approach of crosslinking hemoglobin with superoxide dismutase (SOD) and catalase to form polyhemoglobin-SOD-catalase. We have also used red blood cells hemolysate that contains hemoglobin, SOD and catalase for crosslinking to form polyhemoglobin-SOD-catalase with the same excellent retention of enzyme activities. The main disadvantage is that this only has the same concentration of enzymes as in the red blood cells. Since red blood cells with their enzymes are not effective in severe conditions of ischemia-reperfusion, the direct addition of enzymes at higher concentration would result in a more effective system. Privalle *et al.* has just reported using red blood cell hemoglobin containing SOD and catalase to form pyridoxalated hemoglobin polyoxyethylene. Another approach is Hsia's binding of chemicals with SOD and catalase activities to hemoglobin. Lemon *et al.* is developing a second generation recombinant human hemoglobin. They have substituted a tryptophan into the heme pocket to sterically hinder reactivity with nitric oxide and replacing the distal histidine with a glutamine to improve the oxygen dissociation kinetics. Tuschida and his collaborators in Japan and Rudolph and his collaborators in the U.S. are developing hemoglobin lipid vesicles that are in the advance stages of pre-clinical testing. We are developing a nanoencapsulation approach using biodegradable polylactic acid membrane nanocapsules containing hemoglobin and red blood cell enzymes.

VI-1. TREATMENT OF SEVERE DECOMPRESSION SICKNESS IN SWINE WITH OXYGENT™, A PERFLUOROCARBON EMULSION

D.M. Dromsky¹, A. Fahlman², and B.D. Spiess³. ¹Naval Medical Research Center, Bethesda, MD 20889, ²Carleton University, Ottawa, Ontario, Canada K1S 5B6, ³Medical College of Virginia, Richmond, VA 23298

BACKGROUND: The US Navy seeks adjunctive treatments for severe decompression sickness (DCS) to be used when recompressive treatment is delayed or unavailable. Perfluorocarbon emulsion (PFC) has high dissolving capacity for gases. It has been hypothesized that PFC combined with 100% inspired oxygen (O₂) would improve the outcome in severe DCS.

METHODS: Male Yorkshire swine (N = 45, \bar{x} = 21.3 kg \pm 1.45 SD) were randomized to one of three groups, then compressed to 4.94 ATA on air for 22 hours and brought directly to the surface at 0.91 ATA/min. On reaching 1 ATA, the PFC group received IV infusion of 1 mg/kg methylprednisolone (MP) followed by 6 ml/kg of Oxygent™ (perflubron emulsion, Alliance Pharmaceutical Corp., San Diego, CA.), then breathed 100% O₂. The O₂ group received 1 mg/kg MP, then breathed 100% O₂. The MP group received 1 mg/kg MP, then breathed room air. Outcomes of non-fatal CNS or cardiopulmonary DCS and death were recorded. An earlier group of 12 untreated controls exposed to the same profile was also available for analysis.

RESULTS: Control: N=12, 10 DCS, 7 died. MP: N=15, 14 DCS, 7 died. O₂: N=15, 13 DCS, 6 died. PFC: N=15, 8 DCS, 4 died. The control and MP groups were statistically indistinguishable and therefore combined for analysis. Post-dive O₂ breathing did not significantly reduce morbidity or mortality in this model. The PFC group sustained less DCS (p<0.01) and death (p<0.05).

CONCLUSION: Post-dive treatment with Oxygent™ and 100% O₂ significantly decreased the incidence of severe DCS and death after direct ascent from saturation.

VI-2. LESS PHAGOCYTOSIS OF FLUOROCARBON EMULSION MADE BY FLUOROALKYLATED POLYETHYLENE GLYCOL

Y.-C. Hsu, C.-C. Kwan, and C.-A. Peng. University of Southern California, Los Angeles, CA 90089

Perfluorocarbon (PFC), due to its special feature of dissolving large amount of oxygen, has been investigated for decades as the blood substitute. So far, PFC emulsions have been manufactured based mainly on two surfactants, Pluronic F-68 and egg yolk phospholipids (EYP). However, they have been documented to induce inflammatory or allergic responses when PFC emulsions were injected to the bloodstream of mammals. The cause of these side effects is associated with the phagocytosis of emulsified PFC microparticles by cells such as monocytes and macrophages. In order to lessen the side effects, it is logic to design and develop new surfactants, which are more phagocytosis-resistant and biocompatible. In this study, perfluoroalkylated polyethylene glycol (R_F -PEG) surfactants were synthesized by reacting perfluorooctanoyl chloride ($C_7F_{15}COCl$) with PEG of three different molecular weights: 1450, 4000, and 8000. The purity and interfacial tension of these fluorosurfactants were determined by gel-permeation chromatography and the Wilhelmy plate, respectively. These R_F -PEG surfactants along with Pluronic F-68 and EYP were used to make PFC emulsions separately by an ultrasonic homogenizer. Individual PFC emulsions were then incubated with mouse macrophage J774A.1 cells to examine the degree of phagocytosis. From microscopic observation of cell morphology, our results showed that the process of phagocytosis was retarded by the increment of PEG molecular weight in the perfluoroalkylated PEG surfactants. The interpretation of this qualitative result is that the increase of PEG chain length provides steric hindrance and less protein (opsonins) adsorption. We also harnessed ^{19}F -NMR to quantitatively detect the amount of PFC emulsions phagocytosed by J774A.1 cells. ^{19}F -NMR result is consistent with the qualitative microscopic observation aforementioned. Finally, the hemolytic activity of these synthesized fluorosurfactants were confirmed by no detectable hemolysis of red blood cells at even very high concentrations.

VI-3. PERFLUOROCARBON-STABILIZED INTRAVASCULAR MICROBUBBLES: AN ULTRA-EFFECTIVE MODE OF OXYGEN DELIVERY

Lundgren C., Bergoe G., and Tyssebotn I. Center for Research and Education in Special Environments, and Department of Physiology and Biophysics, State University of NY at Buffalo, New York

Theoretically, microbubbles in the circulation have potential as oxygen carriers, particularly if rapid bubble collapse is counteracted by a poorly permeable gas in the bubbles (1). An intravenously injected 2% emulsion of dodecafluoropentane (DDFPe) (Sonus Pharmaceuticals Inc.) undergoes phase shift at body temperature and generates bubbles which are smaller than a capillary diameter. We demonstrated the feasibility of using such bubbles for O₂ delivery in anesthetized O₂ breathing rats which, in a control series (n=7), were rendered fatally anemic (Hb 2.8g/100 ml) by bleeding and volume replacement with Ringer solution. Similarly treated rats (n=7), which received DDFPe 0.4-0.5 ml/kg (total DDFP dose: 8-10 μ l) survived a drop in Hb to 1.4 g/100 ml and exhibited normal circulatory parameters during 2 hrs of monitoring. Some rats were awakened from anesthesia and exhibited normal behavior; some rats were partially retransfused with autologous blood, exhibiting normal behavior until sacrificed for multiple organ histology after 21 days. No microscopic abnormalities were observed. In another series (n=5) anesthetized O₂ breathing normal rats received 0.02 ml/kg of DDFPe. This increased the PO₂ in abdominal muscle by 58% over a 2-hr period. The generality of the principle that bubbles can serve physiologic gas exchange is further demonstrated by our preliminary experiments with tissue N₂ washout in O₂ breathing pigs. The N₂ yield obtained in 2 hrs in controls was obtained already after 1 hr in animals that had received 0.1 ml/kg b.w. of DDFPe. The DDFPe-derived microbubbles hold promise as a very efficient O₂ delivery vehicle. The projected dosage of fluorocarbon for a given O₂ delivery is less than 1/500 of that of "conventional" fluorocarbon based erythrocyte substitutes.

(1) M.E. Burkhard and H.D. Van Liew. J. Appl. Physiol. 77:2874-2878, 1994.

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VI-4. BIS MALEIDOPHENYL-PEG REAGENTS FOR INTER- MOLECULAR CROSS-BRIDGING (SIZE ENHANCEMENT) OF HEMOGLOBIN A

A.S. Acharya¹, R. Upadhy¹, P.K. Smith², B.N. Manjula¹, and J.M. Friedman¹. ¹Albert Einstein College of Medicine, Bronx, NY and ²BioAffinity Systems, Rockford, IL

Bis-maleidophenyl PEG with PEG spacer chains of the size 0.6K, 2K, 3.4K, 5K and 10K have been designed as site-specific Hb-oligomerizing bifunctional reagents. Contrary to the design strategy, the reaction of oxy HbA (0.5 mM) with a two-fold molar excess of the Bis-Mal-Phe-PEG-2K at pH 7.4 for 1 hr at 23°C resulted in a near quantitative intramolecular cross-bridging of the two Cys-93(Ξ) residues of the protein. Cys-93-ΞΞ-succinimidophenyl HbA is a new class of intramolecularly cross-bridged Hb. The cross-link is outside the central cavity and is exposed to the solvent. Hb concentration had very limited influence on the overall efficiency of this cross-linking reaction. The high efficiency of this intramolecular cross-linking reaction has been rationalized by envisaging that on reaction of one end of the bifunctional reagent with Cys-93(Ξ), the PEG-chain wraps around the tetramer making the other end of the reagent accessible to trans Cys-93(Ξ). If this is the case, changes in the molecular size of the spacer PEG-chain should facilitate intermolecular cross-linking reaction; bifunctional reagents with either shorter or longer PEG-chains may be anticipated to favor intertetrameric cross-linking. The efficiency of Bis-Mal-PEG-0.6K, Bis-Mal-PEG-3.4K, Bis-Mal-PEG-5K and Bis-Mal-PEG-10K to introduce intramolecular cross-linking is 80, 80, 78 and 35% respectively, as opposed to nearly 100% with Bis-Mal-PEG-2K. Except in the case of Bis-Mal-PEG-0.6K, that generates ~20% oligomeric forms of Hb, other PEG-cross-linkers failed to generate much of oligomers. On the other hand, a sample of ∇∇-fumaryl-HbA incubated with Bis-Mal-PEG-0.6K resulted in the oligomerization of more than 60% of the tetramer; the amount of octamer, dodecamer and hexadecamer were present in a decreasing order. The results imply that in the absence of intratetrameric cross-bridges, the intertetrameric cross-bridged species of Hb segregate into intratetrameric cross-bridged species; this is the primary pathway for the generation of the intramolecularly cross-linked HbA in these reactions. Consistent with this, Bis-Mal-PEG-0.6K cross-linking of an equimolar mixture of HbA and HbC, or HbA and HbS generated intramolecularly cross-bridged mixed hybrids. Accordingly, we conclude that Bis-Mal-Phe-PEGs are useful for generation of site-specifically cross-bridged homogeneous oligomeric forms of intramolecularly cross-bridged Hb.

VI-5. ATTENUATION OF HEMOGLOBIN (Hb) NEUROTOXIC POTENTIAL BY CROSS-LINKING WITH ADENOSINE AND CONJUGATION WITH REDUCED GLUTATHIONE (GSH)

J. Simoni¹, G. Simoni¹, D.E. Wesson², J.A. Griswold¹, and M. Feola¹. Departments of ¹Surgery and ²Internal Medicine, Texas Tech University HSC, School of Medicine, Lubbock, TX 79430

During a recent clinical trial, one Hb-based blood substitute was found to cause adverse effects in patients with acute ischemic stroke (*Saxena R., Wijnhoud A.D., Carton H., et al.: Stroke 30:993-6,1999*). Therefore, it has been suggested that the safety evaluation of new blood substitute candidates should also include the study of their neurotoxicity. As a result, we have investigated the neurotoxic potential of our developed red cell substitute, consisting of bovine Hb cross-linked intramolecularly with *o*-ATP and intermolecularly with *o*-adenosine and conjugated with GSH, on cultures of normal human brain neurons and astrocytes. Neurons were obtained from human neural progenitor cells cultured on polyethylenimine coated plates. Astrocytes were used as a third and fourth passage. The effect of this blood substitute used in concentrations of 0.1, 1, 10 and 100 μ M (as a tetramer) was compared with the effects seen with native tetrameric Hb. In the control group, Hb was replaced with plasma. In addition, cells were incubated with both Hb solutions, supplemented with a 10 molar excess of hydrogen peroxide (H_2O_2). The cells were tested for viability (LDH release) and oxidative injury (formation of thiobarbituric acid reactants and the level of intracellular GSH). The astrocytes were also screened for inflammatory reactions by monitoring the activation of nuclear transcription factor NF- κ B and expression of the intracellular adhesion molecule-1 (ICAM-1). Exposure to native Hb resulted in a concentration dependent neuronal injury and the oxidative and inflammatory activation of the astrocytes. The toxicity of native Hb toward human neurons was potentiated by H_2O_2 . Neuronal injury occurred when the concentration of native Hb exceeded 1 μ M, and at 0.1 μ M with the addition of H_2O_2 . The astrocytes demonstrated more resistance than the neurons. The oxidative injury and inflammatory response were observed at a 100 μ M level when the native Hb was oxidatively activated by H_2O_2 . On the contrary, the blood substitute proved to be non-toxic to the neurons and astrocytes at any tested concentration, and it significantly reduced the toxic effect of H_2O_2 . Together, these results indicate that native Hb is extremely toxic to the neurons and this effect is potentiated by H_2O_2 . This data also demonstrates that the chemical modification which combines Hb with adenosine and GSH effectively attenuates this deadly potential.

VI-6. SYNTHESIS AND PHYSICOCHEMICAL CHARACTERIZATION OF Hb-BASED O₂ CARRIERS: COMPARISON BETWEEN CELLULAR AND ACELLULAR TYPES ¹⁾

S. Takeoka, H. Sakai, H. Onuma, and E. Tsuchida. Department of Polymer Chemistry, ARISE Waseda University, Tokyo 169-8555, Japan

A series of Hb-based O₂ carriers were synthesized and their physicochemical characteristics were compared. The acellular type includes intramolecularly crosslinked Hb (XLHb), PEG-conjugated pyridoxalated Hb (PEG-PLP-Hb), hydroxyethylstarch-conjugated Hb (HES-XLHb), and glutaraldehyde-polymerized XLHb (Poly-XLHb). The cellular type is Hb-vesicles (HbV) of which the surface is modified with PEG. Their averaged particle diameters are 7, 22, 47, 68, and 224 nm, respectively, thus all the materials penetrate across membrane filters with 0.4 μ m pore size, though only the PEG-HbV can not penetrate across the filter with 0.2 μ m pore size. PEG-PLP-Hb ([Hb] = 5 g/dL) showed viscosity of 6.1 cP at 332 s⁻¹ and COP of 70.2 Torr which are beyond the physiological conditions (human blood, viscosity = 3 – 4 cP; COP = 20 – 25 Torr). XLHb and Poly-XLHb showed viscosities of 1.0 and 1.5 cp, respectively, which are significantly lower than that of blood. COP of PEG-HbV is regulated to 20 Torr in 5% HSA. HES-XLHb and PEG-HbV/HSA showed a viscosity comparable with human blood. Microscopic observation of human red blood cells (RBC) after mixing with PEG-PLP-Hb or HES-XLHb disclosed aggregates of RBC, a kind of sludge, indicating a strong interaction with RBC, which is anticipated to modify peripheral blood flow in vivo. On the other hand, XLHb and PEG-HbV showed no rouleaux or aggregates of RBC. The acellular Hbs (P₅₀ = 14 - 32 Torr) have their specific O₂ affinities determined by their structures, while that of the cellular PEG-HbV is regulated by coencapsulating an appropriate amount of an allosteric effector (e.g. P₅₀ = 18, 32 Torr). These differences in physicochemical characteristics between the acellular and cellular types indicate the advantages of the cellular type.

1) H. Sakai, M. Yuasa, H. Onuma, S. Takeoka, and E. Tsuchida. Bioconjugate Chem. 2000:11, 56-64.

VI-7. ELECTROCHEMICAL STUDIES OF AN ALBUMIN-HEME HYBRID AS OXYGEN-CARRING HEMOPROTEIN

Y.P. Wu, T. Komatsu, E. Tsuchida. Department of Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555, Japan

We have found that a recombinant human serum albumin (rHSA) incorporating a tetraphenylporphyrinatoiron(II) derivative with a covalently bound proximal base (FeP) (rHSA-FeP) acts as a totally synthetic hemoprotein, which can reversibly bind and release molecular oxygen under physiological conditions as do hemoglobin and myoglobin, and this unusual stability of the oxygenated FeP in aqueous solution is obviously due to the molecular environment around FeP in the albumin.¹⁾ More recently, we have tried to elucidate this phenomenon by electrochemistry.^{2,3)}

This paper describes for the first time the redox behavior of the rHSA-FeP hybrid in aqueous media, which can be directly evaluated using an edge plane pyrolytic graphite electrode modified with didodecyldimethylammonium bromide. The redox behavior of the rHSA-FeP complex fits well with the thin-film electrochemistry, and the redox potential ($E_{1/2}$) shifts to the anodic side with an increase in pH due to the change of its electron density. The counter anions and their concentration also affect the $E_{1/2}$. Compared with that of the naked FeP in aqueous solution, the $E_{1/2}$ of rHSA-FeP shifts in the cathodic direction. This indicates that the hydrophobic environment in the albumin host makes the Fe(II) state difficult to oxidize. An increase in the number of the combined FeP molecule(s) in rHSA from 1 to 4 and 8 results in a positive shift of the $E_{1/2}$. The first bound FeP probably sits at the outer domain of the albumin structure, and the other seven FeP molecules are in a relatively inner position.

E. Tsuchida, T. Komatsu, Y. Matsukawa, K. Hamamatsu and J. Wu, *Bioconjugate Chem.* 10: 797-802, 1999.

Y. P. Wu, T. Komatsu and E. Tsuchida, *Chem. Lett.* 2000 : 1194-1195, 2000.

Y. P. Wu, T. Komatsu and E. Tsuchida, *Bioconjugate Chem.* in press.

VI-8. NO-BINDING PROPERTIES OF RECOMBINANT HUMAN SERUM ALBUMIN INCORPORATING SYNTHETIC HEME (ALBUMIN-HEME)

T. Komatsu, Y. Matsukawa, and E. Tsuchida. Department of Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555 Japan

Several types of hemoglobin(Hb)-based O₂-carriers are currently being studied in clinical trials. Administration of these cell-free Hbs as red cell substitutes, however, often induces acute increases in systemic blood pressure by vasoconstriction. This side-effect is now recognized to be due to the fact that small Hb molecules extravasate through the vascular endothelium and react with the endothelial-derived relaxation factor (EDRF), *namely nitric oxide (NO)*, thus inhibiting its vasorelaxing action.

In this paper, the reaction of NO with a synthetic hemoprotein, the recombinant human serum albumin (rHSA) incorporating *eight* tetraphenylporphinatoiron(II) derivatives bearing a covalently linked axial base (FeP) [rHSA-FeP] has been reported. The UV-vis. absorption spectrum of the phosphate buffer solution (pH 7.3) of rHSA-FeP showed maxima at 425 and 546 nm upon the addition of NO. The carbonyl rHSA-FeP, in which FePs are six-coordinate CO-adducts, also moved to the same species after bubbling with NO gas. ESR spectroscopy revealed that the incorporated FePs in the albumin formed six-coordinate nitrosyl complexes; the proximal imidazole moiety does not dissociate from the central iron when NO binds to the trans side. The NO-binding affinity of rHSA-FeP ($P_{1/2}^{NO}$: 1.9×10^{-6} Torr, pH 7.3, 298 K) was significantly lower than that of FeP itself ($P_{1/2}^{NO}$: 1.8×10^{-8} Torr in toluene).¹⁾ Kinetically, this arises from the decreased association rate constant (k_{on}^{NO}).²⁾ Since NO-association is diffusion controlled, incorporation of the synthetic heme into the albumin matrix appears to restrict the NO access to the central iron(II).

- 1) T. Komatsu, Y. Matsukawa and E. Tsuchida. Chem. Lett. 2000: 1060-1061, 2000.
- 2) T. Komatsu, Y. Matsukawa and E. Tsuchida, Bioconjugate Chem. 12, 2000, in press.

VI-9. HUMAN ERYTHROCYTES CONTAINING EXCLUSIVELY α -NITROSYL HEMOGLOBIN: A PROMISING BLOOD TRANSFUSANT CANDIDATE

A. Tsuneshige and T. Yonetani. Dept. of Biochemistry & Biophysics, University of Pennsylvania Medical Center, Philadelphia, PA 19104

Previous studies on the α -nitrosyl derivative of human adult hemoglobin (HbA), that is, tetrameric Hb in which only the two α -subunits were ligated with nitric oxide (NO), have revealed that under acidic conditions and/or presence of allosteric effectors, this derivative shows extremely reduced affinity for oxygen [Yonetani et al., *J. Biol. Chem.* 273, 20323-20333, 1998]. In this study, we have prepared intraerythrocyte α -nitrosyl Hb (α -NO RBC), by incorporating in a well-controlled manner NO into intact human erythrocytes to a 50% saturation of hemes and exclusively bound to α -subunits, which were confirmed by EPR. Oxygenation studies revealed that α -NO RBC's show also reduced oxygen affinity and diminished Bohr effect (i.e., pH-induced oxygen affinity change). It was found that despite its halved oxygen carrying capacity (since both the α -subunits are already ligated with NO, and only the two β -subunits are available for oxygen binding), α -NO RBC's can efficiently deliver oxygen to tissues under normal physiological conditions, making this a good candidate for a blood transfusant. Supported by NIH HL14508.

Abstracts of Poster Presentations

**PO-1. SYNTHESIS AND IN VITRO ANTIOXIDANT ACTIVITY OF A
POLYMERIZED HEMOGLOBIN-TROLOX CONJUGATE**

J.G. Adamson, G.A. McIntosh, J.B. Stewart, L.T. Wong. Hemosol Inc., Toronto,
Canada M9W 4Z7

Reperfusion of ischemic tissue with oxygen-containing fluid results in formation of oxygen radicals believed to cause the tissue damage underlying ischemic and reperfusion injury (RI). To explore the ability to attenuate this response, Trolox (TX), a water-soluble vitamin E analog, was conjugated to o-raffinose-polymerized Hb (polyOR-Hb) to provide an HBOC with antioxidant properties. TX was linked to Hb or polyOR-Hb through amide linkages between lysine sidechains and the Trolox C2-carboxylate using a water-soluble coupling agent. Up to four TX molecules were attached to each Hb, as measured by reversed phase HPLC-MS. PolyOR-Hb-TX was prepared by direct modification of polyOR-Hb, or by o-raffinose-mediated polymerization of Hb-TX conjugate. The P50 of polyOR-Hb remained at 40 mm Hg (37°C) when TX was conjugated in the absence of oxygen. TX attachment in CO resulted in a P50 of 16 mm Hg. Antioxidant activities of conjugates and controls were measured *in vitro* according to Miki *et al.* (Arch. Biochem. Biophys. 258:373-380, 1987). Activity was measured by delay in onset of, and decrease in extent of, Hb release caused by peroxyl-mediated human red blood cell (RBC) membrane damage. Protection against lysis by Trolox alone (0.5 mM in RBC suspension), Hb (0.2 mM), and Hb (0.2 mM) plus TX (0.5 mM) was 12, 65 and 72%, respectively, suggesting additive antioxidant effects of Hb and TX. A Hb-TX conjugate containing 0.2 mM Hb and 0.4 mM conjugated TX provided 96% protection, indicating a synergistic effect through conjugation. In a separate analysis, a conjugate containing 0.2 mM polyOR-Hb provided 100% protection, compared to 30% and 53% protection by Trolox (0.52 mM) and polyOR-Hb (0.2 mM) alone, respectively. TX conjugation did not alter the hemodynamic properties of polyOR-Hb; mean arterial pressure of conscious rats increased from 101±4 and 110±3 to 142±7 and 151±1 mm Hg after bolus infusion of 0.45 g/kg of polyOR-Hb and polyOR-Hb-TX, respectively. Studies directed at evaluating the *in vivo* antioxidant activity of this HBOC-antioxidant are underway.

PO-2. HAEMOGLOBIN (*ERYTHROGEN*TM)-ENHANCED MICRO-CALLUS FORMATION FROM PROTOPLASTS OF INDICA RICE (*ORYZA SATIVA L.*)

M. Al-Forkan, P. Anthony, J.B. Power, M.R. Davey, and K.C. Lowe. School of Life & Environmental Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

The beneficial effects on the mitotic division of cell suspension-derived protoplasts (wall-less plant cells) of Indica rices (*Oryza sativa L.*) cvs. BR26 and Bini have been studied following supplementation of liquid culture medium with 1:100-1:500 (v:v) of a stabilised, bovine haemoglobin-based (103 g l^{-1}) solution (*Erythrogen*TM; Biorelease Corporation, Salem, USA). Protoplasts were dispersed in the liquid medium, at densities of 1.5×10^6 or $2.5 \times 10^6 \text{ ml}^{-1}$, on nitrocellulose membranes overlaying semi-solidified medium that was supplemented with both *Erythrogen*TM and nurse (feeder) cells of *Lolium multiflorum*. The mean final plating efficiencies (FPEs), as assessed after 28 d of culture, of regenerating rice protoplasts (cv. BR26) cultured with 1:200 (v:v) *Erythrogen*TM at $1.5 \times 10^6 \text{ ml}^{-1}$ ($0.018 \pm 0.003\%$; $n = 8$) and $2.5 \times 10^6 \text{ ml}^{-1}$ ($0.016 \pm 0.002\%$; $n = 8$), were both significantly ($P < 0.05$) greater than controls lacking *Erythrogen*TM ($0.0058 \pm 0.002\%$; $n = 8$ and $0.0041 \pm 0.001\%$; $n = 8$, respectively). Similarly, the mean FPEs of cv. Bini protoplasts cultured with 1:200 (v:v) *Erythrogen*TM at $1.5 \times 10^6 \text{ ml}^{-1}$ ($0.012 \pm 0.003\%$; $n = 6$) and $2.5 \times 10^6 \text{ ml}^{-1}$ ($0.017 \pm 0.001\%$; $n = 6$) were also significantly ($P < 0.05$) greater than their respective controls ($0.003 \pm 0.001\%$, $n = 6$ and $0.002 \pm 0.001\%$, $n = 6$). In contrast, supplementation of the semi-solid medium with 1:100 or 1:500 (v:v) *Erythrogen*TM did not result in sustained mitotic division and microcallus formation in both rice cultivars. These results indicate that supplementation of aqueous-based cell culture medium with respiratory gas-carrying fluids provides a convenient option for enhancing gas supply to cultured cells and tissues of species of biotechnological importance.

PO-3. MODIFIED PORCINE HAEMOGLOBIN HYPERPOLYMERS AS HYPO-ONCOTIC ARTIFICIAL OXYGEN CARRIERS

W.K.R. Barnikol, O. Burkhard, H. Poetzschke, U. Domack, S. Dinkelmann, S. Guth, B. Fiedler, and B. Manz. Clinical Physiology, Witten-Herdecke University, and SanguiBioTech AG, Witten, Germany

Iso-oncotic oxygen carrying plasma expanders for clinical use ('blood substitutes'), as developed worldwide with large efforts, are designed for combating oxygen deficiencies caused by blood losses. They are not suitable for alleviating anaemias, local ischaemias and their complications, like stroke or myocardial infarction, or for use in pre-treatment tumour oxygenation. For this indications, only a hypo-oncotic artificial oxygen carrier – a so-called 'blood additive' – is useful. We have chosen porcine haemoglobin as the basic material for the development of such an oxygen carrying blood additive, because of its inexhaustible availability, as well as similarity in structure and functional behaviour, as compared with human haemoglobin.

To get an hypo-oncotic oxygen carrying blood additive, besides further requirements, haemoglobin molecules must be highly polymerised to polymers with narrow distributions of molecular weights around 1,000,000 g/mol, preferably in high yields and at low costs. But, polymerising haemoglobin by cross-linking normally results in a so-called percolation distribution of molecular weights, with a large amount of unsoluble material, and only poor yields of desired molecular species.

A new one-vessel synthesis procedure, comprising a controlled dilution of the reaction medium during cross-linking of the haemoglobin, avoids the mentioned detrimental reaction progress. Syntheses are easy and cheap to perform at large scale, resulting in yields around 30 % of high molecular polymers, which are suitable for an artificial blood additive. The oxygen pressure at half saturation of these hyperpolymers ranges from 19 to 24 Torr, and Hill's index from 1.8 to 2.2. They are fully compatible with human blood plasma, and at the intended therapeutical concentrations around 30 g/L, their impact on oncotic pressure is very low, and tolerable regarding blood viscosity.

**PO-4. SHEAR-INDUCED PARTICLE DIFFUSION IN VENULES
CALCULATED FROM RED BLOOD CELL DISPERSIONS**

Bishop, J.J.¹, Popel, A.S.², Intaglietta, M.¹, and Johnson, P.C.¹ ¹Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093, ²Dept. of Biomedical Engineering, Johns Hopkins University, Baltimore, MD 21205

A large number of studies have shown that the transport of solutes within a concentrated suspension of particles can be augmented due to the effects of shear-induced dispersive particle migrations. In the case where the suspension is blood, these self-diffusion coefficients have been estimated from previous *in vitro* studies of blood flow in small glass tubes which show that red blood cells exhibit significant erratic deviations in radial position in the laminar flow regime. The purpose of the present study was to assess the magnitude of variability in radial position and velocity for red blood cells flowing in venous microvessels. Using a gated image intensifier and fluorescently-labeled red blood cells in tracer quantities, we obtained multiple measurements of red blood cell radial and longitudinal position at time intervals as short as 5 ms within single venules (45 - 75 μ m) of the rat spinotrapezius muscle. Over the velocity range of 0.3 - 14 mm/s, the root mean square radial deviation was $2.14 \pm 1.23 \mu$ m. Corresponding variations in instantaneous velocity of $\sim 16\%$ can be explained by the shifting of cells to different radial positions along the velocity profile. Induction of red blood cell aggregation by adding Dextran 500 to the blood significantly lowered both the coefficient of variation of velocity and the root mean square radial deviation. Self-diffusion coefficients determined from dispersions of this magnitude are several orders of magnitude larger than the diffusion coefficients due to Brownian motion and could have significant effects on the transport of solutes within the blood stream.

PO-5. RESUSCITATION FROM SEVERE HEMORRHAGE WITH CONJUGATED HEMOGLOBIN: CONSEQUENCES ON SKELETAL MUSCLE OXYGEN TENSION, LACTATE AND ADENOSINE CONCENTRATIONS IN RABBITS

C. Boura¹, A. Caron¹, D. Longrois², P.M. Mertès², P. Labrude¹, and P. Menu¹.
¹University Henri Poincaré-Nancy 1, 54001 Nancy, ²Department of Anesthesia, CHU Nancy, 54511 Vandoeuvre-lès-Nancy, France

Resuscitation from hemorrhagic shock aims at restoring O₂ transport and delivery by increasing cardiac output and arterial O₂ tension. Nevertheless, the restoration of these parameters does not vouch adequate tissue O₂ supply. Depending on the type of plasma expander – colloid or crystalloid - used to restore volemia, tissue O₂ supply may be modulated in virtue of effects on microcirculation and blood rheology. In a rabbit model of resuscitation from severe hemorrhage (50% blood loss), we compared the effects of dextran-benzene-tetracarboxylate-conjugated hemoglobin (Hb-Dex-BTC) on systemic hemodynamics, skeletal muscle O₂ tension (PtiO₂), extracellular concentrations of lactate and adenosine, with those elicited by albumin, hydroxyethyl starch or saline. These parameters were assessed through the combined implantation of a microdialysis probe and a sensitive O₂ electrode into the hind leg. Hemorrhagic shock induced a 50% decrease in mean blood pressure and PtiO₂, and an increase in both extracellular metabolites measured. Following restoration of blood volume with Hb-Dex-BTC, blood pressure increased (12.5%), PtiO₂, lactate and adenosine concentrations returned immediately to basal values. Restoration from hemorrhage with the plasma expanders did not rise blood pressure to pre-hemorrhage values. Tissue and cell responses were different according to the type of plasma substitute: colloids induced a rapid increase in PtiO₂ (similar to Hb-Dex-BTC) but did not impair lactate and adenosine increase. Conversely, PtiO₂ values did not return to pre-hemorrhage level with saline. Combined microdialysis and O₂ sensing techniques indicate that Hb-Dex-BTC improved tissue and cell oxygenation following severe hemorrhage, compared to plasma substitutes. This model may be applied to evaluate the tissue and cell responses with various hemoglobin solutions.

PO-6. A PROJECT FOR THE PRODUCTION AND EVALUATION OF PERFLUOROCARBON-BASED INJECTABLE OXYGEN CARRIERS: PROJECT UPDATE

J.C. Briceño^{1,2}, I.E. Rincón¹, J.F.Vélez¹, I. Castro¹, E. Chavarro^{1,2}, and P. Cabrales^{1,2}.
¹Fundación Cardio-Infantil Instituto de Cardiología, and ²University of Los Andes

For the last few years a research group at our institutions has been working on a project for the production and evaluation of perfluorocarbon-based injectable oxygen carriers (PFC-OCs). In the initial phase of the project, cardiopulmonary bypass (CPB), hemorrhagic shock (HS), and acute normovolemic hemodilution (ANH) were identified as the procedures on which PFC-OCs were more suitable for use. Research efforts were then concentrated on studying the effects of the use of PFC-OCs on animal models of CPB, HS and ANH. Comprehensive information about oxygen transport and consumption, hemodynamics, blood gasimetry, blood chemistry, fluid balance, and secondary effects associated with the use of a PFC-OC (Oxyfluor™, HemaGen/PFC, St. Louis, MO) in those procedures was collected and analyzed.

In April 2000, a two-year grant from the Colombian Science Foundation (Colciencias) was awarded to pursue research on PFC-OCs. The objectives of the project are: 1) Building of the lab facilities with all the technical requirements for sterile production of PFC-OCs; 2) Selection and purchasing of equipment for production of PFC-OCs (i.e., microemulsifier, particle analyzer); 3) Selection and purchasing of raw materials for production of PFC-OCs (i.e., perfluorocarbons, emulsifiers, stabilizers); 4) Detailed design of the preformulations of PFC-OCs, and of the stability, safety and efficacy tests; 5) Production and evaluation of preformulations and selection of the optimal formulation; 6) In vivo testing of the formulation in animal models of CPB, HS and ANH; and 7) Technology transfer to the pharmaceutical industry, including design of clinical trials and scaling up of manufacturing facilities.

PO-7. CHAGAS DISEASE: RISK OF TRANSFUSION-TRANSMISION IN ENDEMIC COUNTRIES AND POTENTIAL RISK IN NON-ENDEMIC COUNTRIES

P. Cabrales, and J.C. Briceño. Fundación Cardio-Infantil Instituto de Cardiología, and University of Los Andes

Chagas disease, a vector-borne disease caused by the parasite *Trypanosoma cruzi*, is a serious health problem for the population of South and Central America. Blood transfusion is one most common way in which this disease is transmitted. Five cases of *T. cruzi* transmission from transfusions have been reported in North America ¹. Movement of transfusable blood and blood components between countries is relatively uncommon. However, infectious agents can cross international borders through immigration or travel from regions of high incidence. The immigration of millions of persons from *T. cruzi*-endemic areas and increased international travel have raised concerns about the potential for transfusion-transmitted Chagas disease in vector-free countries. This report analyses data from 1993 to 1996 screening of blood donors from eight countries of Central and South America ². The data show a great number of contagions of *T. cruzi* caused by blood transfusions (see table). *T. cruzi* can be disseminated to other countries where blood screening for *T. cruzi* is not accomplished.

	No. Donors	T. Cruzi	Ratio: infections:donations
Bolivia	37948	832	1:46
Chile	217312	236	1:921
Colombia	352316	875	1:403
Ecuador	98473	20	1:4924
El Salvador	48048	85	1:565
Guatemala	45426	33	1:1377
Nicaragua	46001	10	1:4600
Paraguay	32893	41	1:802
Perú	52909	393	1:135
Venezuela	204316	57	1:3584

Estimates of transfusion-transmitted infectious diseases, by country[2].

This analysis indicate the necessity for long-term screening of blood bank donors to reduce the risk of transfusion transmission of the disease even in countries where the vector is not present.

1 Wendel S., Transfusion-transmitted Chagas' disease., Curr Opin Hematol. 1998 Nov;5(6):406-11.

2 Workshop over blood quality control in transfusions, Pan-American Health Organization, 1996.

PO-8. PLURONIC F-68 ENHANCED SHOOT REGENERATION IN A POTENTIALLY NOVEL CITRUS ROOTSTOCK

G.O. Cancino, M.I.S. Gill, P. Anthony, M.R. Davey, J.B. Power, and K.C. Lowe.
School of Life & Environmental Sciences, University of Nottingham, University Park,
Nottingham NG7 2RD, UK

Supplementation of aqueous-based culture media with low concentrations of non-ionic surfactants enhances sustained mitotic division of cells *in vitro*. For plant cells, the use of surfactant supplements is of significant biotechnological importance since such components improve micropropagation systems. In the present investigation, the effects have been studied *in vitro* of the polyoxyethylene-polyoxypropylene block copolymer surfactant, Pluronic® F-68 (Poloxamer 188), on shoot regeneration and bud induction in cultured epicotyl and cotyledon explants of *Citrus depressa*, a potential alternative rootstock to *C. jambhiri* for commercial *Citrus*. Supplementation of Murashige and Skoog-based, agar-solidified shoot regeneration/bud induction (SRBI) medium (1) with 1.0 mg l^{-1} 6-benzylaminopurine and 0.5% (w/v) of a commercial grade of Pluronic® F-68 (Sigma, U.K.) significantly ($P < 0.05$) increased the mean fresh weight of explants and regenerating shoots by a maximum of 60%, the proportion of explants exhibiting shoot/bud regeneration by 25% and the mean number of shoots per epicotyl explant by 184%, compared to untreated controls. Similarly, 0.5% (w/v) Pluronic® F-68 significantly ($P < 0.05$) enhanced the mean percentage bud induction (91%) and the number of buds regenerated (>4-fold) per cotyledon explant. Interestingly, the mean fresh weight gain for both explant types was unaffected across the range of concentrations (0.001-0.1% w/v) of Pluronic® F-68 evaluated. Regenerated plants from epicotyl explants were readily acclimatized to glasshouse conditions. Overall, these results indicate that Pluronic® F-68 as a medium supplement enhances morphogenesis in *Citrus* rootstocks.

PO-9. IDENTIFICATION BY E.P.R. SPECTROSCOPY OF THE FREE RADICAL REACTIONS ASSOCIATED WITH THE INFUSION OF CROSS-LINKED HEMOGLOBIN TO EXCHANGE-TRANSFUSED RABBITS

A. Caron¹, J. Dunn², P. Menu¹, A. Alayash³, M.T. Wilson² and C.E. Cooper².
¹University Henri Poincaré-Nancy 1, 54001 Nancy, France, ²University of Essex, Colchester CO4 3SQ, United Kingdom, ³Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892

The reaction between ferric heme proteins and peroxides results in the formation of ferryl iron and a globin-associated cationic free radical which can be detected by Electron Paramagnetic Resonance (EPR) spectroscopy^{1,2}. These reactions are important because they may play a role in mediating the toxic side-effects of HBOCs. In a rabbit model of 20% exchange transfusion with bis(3,5-dibromosalicyl)fumarate crosslinked hemoglobin ($\alpha\alpha$ -Hb), both arterial and venous, plasma and whole blood samples were collected to determine the extent of free radicals associated with hemoglobin infusion. The major findings from the EPR spectroscopy analysis were: i) methemoglobin in the plasma increased upon transfusion of $\alpha\alpha$ -Hb, but then declined with a half time of approximately 1 hour; ii) immediately following transfusion the iron content of serum transferrin increased by approximately 25% - there was no further change; iii) strikingly, the concentration of the globin radical in the whole blood remained constant throughout the experiments. No globin radical was detectable in plasma. These results indicate a slow methemoglobin reduction via a reductive process at the red cell membrane. The increased iron content of serum transferrin may be due to a small free iron content in the $\alpha\alpha$ -Hb formulation or to accelerated autoxidation process and/or heme breakdown products of $\alpha\alpha$ -Hb. The lack of a globin-associated radical in the plasma suggests there is a lower rate of hydrogen peroxide formation than in the red blood cell, or the presence of reductant(s) reducing the radical.

PO-10. ROLE OF FORMAL REDUCTION POTENTIAL OF HEMOGLOBIN-BASED OXYGEN CARRIERS IN METHEMGLOBIN REDUCTION BY PLASMA COMPONENTS

S.C. Dorman¹, C.F.Kenny¹, R.E. Hirsch² and J.P. Harrington¹. ¹Dept. of Chemistry, University of South Alabama, Mobile AL., ²Department of Medicine, Albert Einstein College of Medicine, Bronx, NY

A functional requirement for all hemoglobin-based oxygen carriers (HBOC) is the maintenance of the heme-iron in the reduced state. This is necessary for the reversible binding/release of molecular oxygen and minimizing levels of methemoglobin. Acellular hemoglobins are particularly susceptible to oxidation and denaturation. In the absence of the intrinsic reducing systems within the red blood cell, the reduced heme-Fe⁺² can be oxidized to form increasing levels of metHb that give rise to free radicals and oxidative cellular damage. If acellular HBOC are to be utilized as red cell substitutes for oxygen delivery, these carriers must be stabilized in the plasma, the carrier medium. Normal plasma contains reducing components, such as ascorbic acid and glutathione that may afford protection to these acellular HBOC through electron-transfer mediated processes. For these components to provide effective reduction of an HBOC, a favorable reduction potential difference must exist between the reducing agent and the HBOC. Using a modified thin-layer spectroelectrochemical approach, a determination of the formal reduction potential (vs Ag/AgCl) of several oxygen carriers, including monomeric myoglobin, tetrameric HbA and HbS, cross-linked HBXL99 α , polymerized oxyglobin, and the naturally available multimeric *Lumbricus* hemoglobin, have been determined. In contrast to the negative formal reduction potentials (-155 to -50 mV) obtained for Mb, HbA, HbS, cross-linked HbXL99 α , and oxyglobin, LtHb exhibited a positive formal reduction potential (~100 mV). This may help explain the greater effectiveness of such reducing agents as ascorbic acid, glutathione and β -NADH to reduce met LtHb, compared to the other met HBOC, back to the required reduced form necessary for physiological binding/release of oxygen. Each component was capable of reducing met LtHb to the fully reduced state, although the kinetics of these reductions were different. This suggests that consideration must be given to those factors affecting the redox properties of any newly designed HBOC.

PO-11. HEMOGLOBIN - ADENOSINE - GLUTATHIONE COMPLEX AS A RED CELL SUBSTITUTE. IS THIS A PERFECT MATCH?

M. Feola and J. Simoni. Department of Surgery, Texas Tech University Health Sciences Center, School of Medicine, Amarillo, TX 79106 and Lubbock, TX 79430

The development of a hemoglobin (Hb)-based red cell substitute has made significant progress during the past two decades with the resolution of problems related to Hb's purity, oxygen affinity and molecular size and stability. However, new problems have been uncovered, related to the vasoconstrictive, pro-oxidant and pro-inflammatory properties of Hb, which have not been generally addressed. Toward resolution of these problems we have reacted bovine Hb with *o*-adenosine 5'-triphosphate (*o*-ATP), *o*-adenosine and reduced glutathione (GSH). The idea behind the use of *o*-adenosine was to counteract the vasoconstrictive and pro-inflammatory properties of Hb with the activation of adenosine A2 and A3 receptors, which would produce vasodilation and moderation of inflammatory reactions. The additional idea of conjugation of Hb with GSH was to introduce more electronegative charges onto the surface of the Hb molecule, which would make it less "visible" to phagocytes and would block its transendothelial passage. At the same time, GSH would "shield" the heme from the reactive oxygen species and nitric oxide, therefore lowering the Hb pro-oxidant and vasoconstrictive potential. Secondly, we expected the reaction with *o*-ATP to stabilize Hb tetramers and prevent its dimerization, and the reaction with *o*-adenosine (affinity cross-linker) to allow the formation of Hb-oligomers and avoid the formation of toxic high molecular weight polymers. The U.S. and foreign patents cover the manufacturing and clinical applications of this red cell substitute. Experiments *in vitro* and *in vivo* have verified the expectations of this formulation. The results of these experiments are being presented separately.

PO-12. MOLECULAR ENGINEERING OF A HOMOGENEOUS POLYMER OF TETRAMERIC HEMOGLOBINS

Fronticelli, C.^{1,2}, Arosio, D.¹, Bobofchak, K.M.², and Vasquez, G.B.³ ¹University of Maryland Medical School, Baltimore, MD, 21201, ²Johns Hopkins University Medical School, Baltimore, MD, 21287, ³Center for Advanced Research in Biotechnology and National Institute of Standard and Technology, Rockville, MD, 20850

We have engineered a recombinant mutant human hemoglobin, Hb Prisca Ξ (S9C+C93A+C112G), which assembles in a polymeric form. The polymerization is obtained through the formation of intermolecular S-S bonds between cysteine residues introduced at position Ξ 9, on the model of Hb Porto Alegre (Ξ 9Ser 6Cys)(Bonaventura and Riggs Science 155: 800-802, 1967). C Ξ 93 and C Ξ 112, were replaced in order to prevent formation of spurious S-S bonds. Dynamic light scattering measurements indicate that the polymer is a homogeneous population comprised of 6 to 8 tetrameric hemoglobin molecules. In the presence of strong reducing agents the polymer reverts to its tetrameric form. During the depolymerization process a direct correlation is observed between the hydrodynamic radius and the mass of the protein. We interpret this to indicate that the hemoglobin molecules are tightly packed in the polymer with no empty spaces. The tight packing of the hemoglobin molecules suggests that the polymer has a globular shape and thus allows estimation of its radius. A possible globular arrangement of a finite number of tetrameric hemoglobin molecules is presented. The conformational and functional characteristics of this polymer, such as heme pocket conformation, stability to denaturation, autooxidation rate, oxygen affinity and cooperativity, remain similar to those of tetrameric human hemoglobin.

At present we do not know to what extent the S-S bonds are resistant to reducing agents present in the plasma if this product is injected in circulation. These bonds are only 5 Å long, thus it is reasonable to think that they should be sterically protected by the large size of the molecules.

PO-13. FFF & SPLITT TECHNIQUES FOR SIZE-BASED FRACTIONATION / CHARACTERIZATION OF BLOOD RELATED SPECIES

M.E. Hansen¹, Y. Jiang¹, L. Moore², and M. Zborowki², ¹FFFractionation, LLC, Salt Lake City, UT 84118, ²Dept. of Biomedical Engineering, Cleveland Clinic, Cleveland, OH 44195

FFF and SPLITT are relatively unknown techniques, yet these techniques are ideally suited for separation / characterization of biological samples such as blood cells, emulsions, and drug delivery vehicles. Both techniques provide high resolution separation under gentle, low shear conditions. Field-Flow Fractionation (FFF) is an analytical-scale, elution-based technique providing size information along with the separation. Split-flow thin cell fractionation is also based on flow through a channel. However, SPLITT complements FFF as it is a semi-preparative scale technique operating in a continuous vs batch mode.

FFF has been used to characterize the droplet size distribution of perfluorocarbon emulsions (D.H. Klein, D.B. Burtner, L.A. Trevino, R.A. Arlauskas. *Biomat. Art. Cells & Immob. Biotech.* 20: 859-864, 1992). Since the FFF process generates size-based fractions as well as the size distribution information, fractions of known sizes can be collected and submitted to further analysis to, for example, determine the chemical composition of each fraction. This capability enabled a study of Ostwald ripening of an artificial blood substitute (R.A. Arlauskas, D.H. Klein, J.G. Weers. *Cells, Blood Subs., Immob. Biotech.* 22:1317-1323, 1994).

SPLITT fractionates the sample into two fractions: one larger & one smaller than the cut-off diameter. By varying flow conditions the cut-off diameter can be varied so that narrow size cuts are generated. We studied SPLITT capabilities for fractionating white blood cells & found that these cells had a bimodal distribution, characteristic of lymphocytes and monocytes. Thus, the separation capabilities of the SPLITT technique were confirmed by separating lymphocytes from monocytes

This presentation will provide more detail about the FFF and SPLITT techniques. The studies mentioned above will also be described, as well as the use of FFF for other relevant samples, such as liposomes, compacted DNA, and aggregated protein species.

PO-14. CROSS-LINKING OF HEMOGLOBIN A₀ WITH *O*-RAFFINOSE: OXYGEN TRANSPORT AND REDOX CHEMISTRY

Y. Jia¹, F. Wood¹, R.E. Cashon², and A.I. Alayash¹. ¹Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda MD 20892, ²Department of Biochemistry and Molecular Biology, University of Maine, Orono MA 04468-5735, USA

O-R-polyHbA₀, an intra- and intermolecularly cross-linked derivative of human HbA₀, was found to have lower oxygen affinity, a reduced Bohr effect and no measurable cooperativity ($n_{\text{max}}=1$). In addition, we found that the enthalpy change (ΔH) associated with oxygen binding is reduced by a factor of two in the modified protein. The kinetics of O₂ and CO binding to the proteins were examined by rapid mixing techniques. In unmodified HbA₀, inositol hexaphosphate (IHP) causes an increase in oxygen dissociation rate (k_{off}) and a decrease in CO association rate (k_{on}). Neither of these allosteric effects were seen in parallel studies with *O*-R-polyHbA₀. The apparent lack of T→R transition seen with *O*-R-polyHbA₀ was confirmed by circular dichroism (CD) studies, as reflected by absence of changes in the Soret region of the CD spectrum (~420 nm) upon the addition of IHP. This may be contrasted with a 2-fold increase in ellipticity as well as a shift to a longer wavelength typically seen with HbA₀. The rate of NO-induced oxidation of the oxy form was slightly reduced in the *O*-R-polyHbA₀ compared to that of HbA₀. Titration of the thiol groups indicates that β Cys-93 of *O*-R-polyHbA₀ is either inaccessible in the modified protein or blocked by the *O*-raffinose modifying groups. Autoxidation studies at 25°C revealed biphasic kinetics for both proteins. In the initial phase, HbA₀ autoxidizes at a rate 2.5 times faster than the polymerized protein. However, in the second, slower oxidative phase, *O*-R-polyHbA₀ autoxidized 1.6 times faster than HbA₀. Rapid mixing of the Fe³⁺ form of the proteins with H₂O₂ resulted in an increased rate of Fe⁴⁺ iron formation of the modified protein. These results indicate that locking HbA₀ in the T-conformation with *O*-raffinose may compromise its ability to effectively deliver oxygen and increase its susceptibility to oxidative damage.

PO-15. HEMOMAX – A NOVEL CONCEPT FOR RESUSCITATION AFTER SEVERE HEMORRHAGIC SHOCK

Kamemevamov

HemoMax is a new preparation, based on plant-derived polysaccharides, which is designed for the improvement of impaired blood circulation. We studied the effect of HemoMax infusion on hemodynamics after severe hemorrhagic shock. Hemorrhage was induced in anesthetized rats (n=40) by withdrawal of about 50-60% of the animals' blood over 30 min (to a mean arterial pressure of 25 mm Hg). The bleeding was followed by a 10 min period of uninterrupted hemorrhagic shock and then resuscitation with a plasma expander. Group 1 (test) received Plasma-Lyte containing HemoMax at a concentration of 1-2 $\mu\text{g/ml}$, Group 2 (control) received only Plasma-Lyte. In both groups, no oxygen carrier or assisted ventilation was utilized during the resuscitation. Arterial blood pressure and tissue perfusion were recorded.

PO-16. DEVELOPMENT AND EVALUATION OF LIPOSOME-ENCAPSULATED HEMOGLOBIN NEO RED CELL (NRC) AS AN ARTIFICIAL OXYGEN CARRIER

S. Kaneda, Y. Ogata, T. Kimura, H. Goto, Y. Tsutsui, Y. Asakawa, T. Shizawa, Y. Ishizuka, and A. Takahashi. Terumo Corporation, R&D Center, 1500 Inokuchi, Nakai-machi, Ashigarakami-gun, Kanagawa 259-0151, Japan

We have developed and evaluated a new generation of artificial oxygen carriers (Neo Red Cell:NRC). NRC is the liposome-encapsulated hemoglobin (Hb), constituted of stroma-free hemolysate (SFHL) with the methemoglobin (metHb) reducing enzymatic system and with inositol hexaphosphate as the allosteric effector. We established a high-efficiency production method of liposome-encapsulated Hb and succeeded in drastically lowering the costs of manufacturing.

NRC thus manufactured has an Hb concentration of 6g/dl, a mean diameter of 200nm, an encapsulation efficiency of 1.9g-Hb:1g-lipids and $P_{50}O_2$ of 45-50mmHg.

We have studied the efficacy and safety of NRC in vitro and in vivo. The oxygen transporting efficiency of NRC under respiration with 30-50% oxygen was superior to that of human red blood cells (hRBC) because of its high P_{50} value. NRC showed double efficacy per g Hb in comparison with hRBC for lactic acid and ATP levels in animals subjected to exchange transfusion.

NRC showed long circulation time in kinetics studies, and its half-life in blood on rats and dogs was about 20h and 35h, respectively. The preliminary toxicology evaluation in rats showed that NRC was sufficiently safe ($LD_{50}>100\text{ml/kg}$).

NRC was produced with the metHb reducing enzymatic system in internal solution, and we optimized the composition and concentrations of substrates added to the SFHL. So that NRC could be stable for a long time during storage without metHb generation. Furthermore, generation of metHb in vivo was also reduced with this mechanism, and we estimated that the half-life of NRC's biological activity as oxygen carrier was about 20hrs in dogs.

PO-17. AMELIORATING EFFECTS OF PERFLUBRON EMULSION ON SICKLE RBC-INDUCED VASOOCCLUSION

D.K. Kaul, X.D. Liu, and R.L. Nagel. Albert Einstein College of Medicine, Bronx, NY

In ex-vivo microcirculatory preparations (Kaul et al, PNAS, 1989) as in "in vivo" models (Fabry et al, PNAS, 1989), sickle RBC-induced vasoocclusion is many times partial, allowing for decreased remnant flow. Hence, if oxygen is delivered to these areas, decreased obstruction might be achieved (Kaul et al. JCI, 1995). We have tested the efficacy of an oxygenated perflubron emulsion ([PFE], *Oxygent*TM; Alliance Pharmaceutical Corp., San Diego, CA) for its anti-vasoocclusive effects in the ex vivo mesocecum preparation. Bolus infusion of deoxygenated SS RBC (Hct 30% in plasma; HbO₂, <5%) into the ex vivo preparations (n=5) pretreated with platelet-activating factor (200 pg/ml) caused widespread adhesion of SS RBC, frequent blockage of postcapillary venules, and significant increase ($P<0.05$) in the peripheral resistance (PRU). Following the passage of SS bolus, a persistent microvascular blockage was indicated by a 2-fold elevation in PRU ($P<0.05$). Infusion of control Ringer-albumin solution had no effect on the PRU. Importantly, infusion of PFE (0.3 ml) caused a significant decrease in PRU ($P<0.05$) that approached pre-infusion baseline values. Video-replays showed that the PRU decrease was accompanied by complete dislodgement of trapped sickled RBC from some partially obstructed vessels, but not from completely obstructed vessels. However, there was no effect on adhered SS RBC. Also, in separate experiments, PFE had no effect on vascular tone (n=3).

Rat mesocecum evaluation of fluorocarbon infusion on the peripheral resistance:

Infusion sequence	PRU (mm Hg/ml/min/g)*					Mean \pm SD
	#1	#2	#3	#4	#5	
Pre-infusion	8.6	7.9	8.1	7.8	8.8	8.2 \pm 0.4
Post-SS infusion	11.0	11.9	20.1	18.4	19.2	16.1 \pm 4.3*
Control Ringer	10.3	10.2	18.1	17.5	13.0	13.8 \pm 3.8*
<i>Oxygent</i>	7.9	8.5	9.9	8.5	8.6	8.7 \pm 0.7

*PRU = $P_a - P_v$ (mmHg)/venous outflow (ml/min)/tissue weight (g). P_a = arterial perfusion pressure; P_v = venous outflow pressure. * $P<0.05$ compared with pre-infusion and PFE (Newman-Keuls multiple comparisons).

We conclude, that *Oxygent* is capable of reducing sickle RBC-induced vasoocclusion and that further development of this approach is advisable.

PO-18. ARTIFICIAL RED CELL AS TREATMENT OF ACUTE CEREBRAL INFARCTION: AN EXPERIMENTAL STUDY IN THE RAT

A.T. Kawaguchi¹, M. Haida¹, Y. Ogata², Y. Izumi¹, T. Tutui², and S. Koide¹. ¹Tokai University School of Medicine, Isehara, Japan, ²Terumo Co. Ltd, Tokyo, Japan

Background Treatment of cerebral infarction (CI) has been limited to indirect therapies aiming at reduction of edema or oxygen demand.

Objectives Since artificial red cells of Terumo (Neo Red Cell: NRC) is made much smaller (0.2 μ m) than regular red cell, we hypothesize that NRC may deliver oxygen not only to the peripheral area at risk of CI but also infarcted area *per se* to limit damage or area of infarction.

Methods CI was created by inserting a 2-0 monofilament suture to occlude the middle cerebral artery in SD rats. One minute later, 10 ml of NRC (G1) were infused at a rate of 1 ml/min with the same amount of blood being drawn to avoid volume overload. G1 rats were compared with CI rats with vehicle infusion (G2) and CI rats with no treatment (G3). Severity of edema was compared 24 hours later on T2 weighted images obtained by using 0.5T MRI imaging system (Vectra Fast, GEYMS, JAPAN) by signal strength at cortex, striatum, hippocampus and limbic lobe.

Results Signal strength \pm standard error were tabulated.

	N	Cortex	Striatum	Hippocampus	Limbic Lobe
G1(NRC)	5	107 \pm 5	97 \pm 05	123 \pm 10	121 \pm 07
G2(Vehicle)	6	124 \pm 3	117 \pm 04	128 \pm 05	116 \pm 10
G3(Control)	8	114 \pm 9	119 \pm 11	121 \pm 07	109 \pm 10
G1 vs G2: P=		0.009	0.012	NS	0.043
G1 vs G3: P=		NS	NS	NS	NS
G2 vs G3: P=		NS	NS	NS	NS

Conclusion NRC (G1) significantly reduced brain edema as compared to vehicle infusion (G2). Low oncotic pressure of the NRC and vehicle solution appeared to cause non-significant aggravation of edema in G2 and reduced protective effects of NRC in G1 group.

PO-19. AN ANTI-NITRIC OXIDE STRATEGY IN SEPSIS-MEDIATED VASOPLESIA: A COMBINATORY APPROACH

H.W. Kim, and A.G. Greenburg. Brown University and The Miriam Hospital, Providence, RI 02906, USA

In sepsis, excessive NO production appears to be a primary cause of the refractory hypotension contributing to a high mortality rate. Most current efforts are directed toward maximal NO synthase (NOS) inhibition. Shutdown of NOSs, however, may produce a negative clinical outcome since both constitutive and inducible NOS functions are essential for normal vascular and immune functions. We tested a hypothesis that submaximal NOS inhibition combined with NO scavenging may produce a more desirable outcome. First, isolated thoracic aortic rings from male SD rats (N=6/group) were incubated with lipopolysaccharide (LPS; E. coli 055:B5, 14mg/dl) for 6 hours. The vessel rings were then treated with 2 μ M purified human Hb (a NO scavenger), 6 μ M N^w-nitro-L-arginine methyl ester (NAME; NOS inhibitor), both 2 μ M Hb and 6 μ M NAME (Hb+NAME), or normal saline (control). Norepinephrine (NE) dose-responses were assessed before and after the experimental treatments and mean log median effective doses (logEC₅₀) compared. Second, male SD rats were subjected to sepsis by cecal ligation and puncture (CLP). Twenty four hours later, the animals were cannulated, randomly assigned to one of 4 groups (N=5-6 ea), and given an i.v. injection of 0.5mL bovine serum albumin (BSA, 5g/dL), 0.5mL Hb (7g/dL), 50uL NAME (1M), or both Hb and NAME. Blood pressure (BP), cardiac output, systemic vascular resistance and BP responses to NE (40ng/Kg) (NR) were assessed before and after treatments. Both Hb and NAME treated vessel rings had significantly lower mean logEC₅₀ values compared with untreated control (-10.2 \pm 0.1M and -10.1 \pm 0.2M vs -9.5 \pm 0.4M, P<0.01, t-test). The logEC₅₀ values with Hb+NAME were also significantly improved over control (-10.3 \pm 0.1M, P<0.001) but was not significantly different from Hb or NAME alone (P>0.05, ANOVA/Neuman-Keuls tests). Similarly, septic animals treated with Hb, NAME and NAME+Hb showed significantly improved mean BP and NR compared with control (BSA) group (P<0.05). In conclusion, NO scavenging with Hb, alone or in combination with NO synthesis inhibition, appears to be effective in modulating sepsis mediated hypotension and vasoplegia. Combinatory strategy may allow controlling NO levels in sepsis without shutting down the crucial NOS functions.

PO-20. MYOGENIC TONE AND HEMOGLOBIN VASOACTIVITY IN THE ISOLATED RAT THORACIC AORTA

H.W. Kim, J. Tai, and A.G. Greenburg. Brown University and The Miriam Hospital, Providence, Rhode Island, USA

Endothelial nitric oxide synthase (eNOS) is presumed to be constitutively active in most blood vessels. In isolated rat thoracic aorta, however, hemoglobin (Hb, an avid NO scavenger) does not elicit contraction without prior tone enhancement. To understand this apparent paradox, we investigated relationship between the vascular tone and the vessel ring contractile responses to purified human hemoglobin (Hb). Thoracic aortic rings (6-7/group) prepared from adult male SD rats were arranged for standard isometric tension measurements. While, in unstimulated vessel rings, Hb as high as 4 μ M did not elicit any measurable contractions, Hb as low as 0.1 μ M Hb produced notable contractions following agonist induced tone enhancement. Following adrenergic tone enhancement with 50nM norepinephrine (NE), 2 μ M Hb elicited $21.8 \pm 13.2\%$ (Mean \pm SD) additional contractions ($P < 0.05$, Student's t-test). To determine whether alpha adrenergic activation is the only condition for Hb induced contraction, KCl, a depolarizing agent, was also tested. In vessel rings precontracted with 100mM KCl, 2 μ M Hb also elicited $21.8 \pm 20.1\%$ additional contraction ($P < 0.05$). Phentolamine, an alpha adrenergic antagonist, did not prevent the KCl/Hb induced contractions. Removal of the endothelium or pretreatment with 2 mM N^G -nitro-L-arginine methyl ester (NAME), a NOS inhibitor, prevented the Hb induced additional contractions confirming endothelial NO involvement. When vessel ring tension was passively increased by a mechanical stretch to various levels, 1-4 μ M Hb did not elicit significant contraction. In contrast, when vessel rings were tone enhanced with a low dose of NE, vessel ring tension as low as 0.1g allowed Hb elicited a significant contraction (0.22 ± 0.05 g, $N=6$, $P < 0.05$). In conclusion, an active myogenic tone appears to be required for Hb induced contraction in the isolated rat thoracic aorta. In this vessel type, eNOS may be minimally active in the basal state but upregulated upon agonist induced tone enhancement.

PO-21. TEMPORAL EFFECT OF HEMOGLOBIN RESUSCITATION ON SEPSIS MORTALITY

H.W. Kim, A. Messier, and A.G. Greenburg. Brown University and The Miriam Hospital, Providence, Rhode Island, USA

Purpose: Hemoglobin (Hb)-based oxygen carriers are promising resuscitation fluids for hemorrhage shock. Aside from renal excretion, the reticuloendothelial system (RES) is a primary mechanism for Hb clearance. Infusion of large amounts of Hb-based resuscitation fluid could overwhelm the RES and impair post-resuscitation host defense. We investigated the temporal relationship between hemorrhage-resuscitation and sepsis survival. Methods: Male SD rats were subjected to 30 minute 1/3 blood volume hemorrhage and resuscitated with shed blood volumes of purified human hemoglobin solution (SFH; 7 gHb/dL). Sepsis was induced by cecal ligation and puncture (CLP) 24 hrs before, 0, 24 or 72 hrs after hemorrhage/resuscitation (H/R) with SFH. Results: When H/R was performed concomitantly with CLP, 7-day survival was 67% (N=6). Control animals (CLP without hemorrhage; N=6) showed a 50% survival. When H/R was performed 24 hrs (N=5) or 72 hrs (N=5) before CLP, survival was 100%. In contrast, 0% survival was seen when H/R was performed 24 hrs after CLP (N=5). Survival of animals resuscitated with SFH prior to sepsis induction was significantly higher than other groups ($P < 0.002$, Logrank test). In these animals, mRNA expression for heme oxygenase-1 (HO-1), an inducible isoform, was detected. In addition, hepatic heme oxygenase activity levels measured at 24 hours after CLP were significantly higher ($1.8 \pm 0.3U$ vs $0.9 \pm 0.3U$, respectively, N=3) ($P < 0.02$, Student's t-test). Conclusion: Hb resuscitation does not appear to enhance post-resuscitation sepsis mortality. Rather, Hb resuscitation may improve subsequent sepsis survival if performed concomitantly or prior to sepsis. The improved survival may be mediated through induction of HO-1, a heme catabolizing enzyme and heat shock protein, which may protect the host from inflammation mediated cellular injuries.

PO-22. BENEFICIAL EFFECTS OF PLURONIC F-68 AND ARTIFICIAL OXYGEN CARRIERS ON THE POST-THAW RECOVERY OF CRYOPRESERVED PLANT CELLS

K.C. Lowe, P. Anthony, M.R. Davey, and J.B. Power. School of Life & Environmental Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

The storage of prokaryotic and eukaryotic cells at ultra-low temperature in liquid nitrogen (-196°C) is a procedure that has assumed an increasingly important role in underpinning many aspects of biotechnology and germplasm conservation. For eukaryotic cells, the transition from a cryopreserved state to physiologically normal temperatures and oxygen tensions, induces respiratory imbalances that may stimulate the production of toxic oxygen radicals, which impair cellular functions. Novel treatments that focus specifically on enhancing oxygen delivery to cells are important in maximising post-thaw cellular recovery. Recently, several approaches have been evaluated with higher plant cell suspension cultures as a model, biotechnologically-important, totipotent eukaryotic cell system. Such treatments include exposure to non-ionic surfactants, primarily the polyoxyethylene-polyoxypropylene block co-polymer, *Pluronic*® F-68 (Poloxamer 188), and artificial oxygen carriers, the latter based on inert perfluorochemical liquids or chemically-modified haemoglobin. Such compounds are used to supplement the culture medium during the post-thaw recovery phase of cell growth. When employed either alone or in combination, such novel treatments stimulate significantly the post-thaw viability and biomass production of cultured plant cells, probably through improvements in intracellular oxygen flux. Such technologies will be exploitable in extending and/or improving cryopreservation protocols for a range of eukaryotic cells.

PO-23. OXYGEN BINDING CURVES OF HEMOGLOBIN AND RED BLOOD CELLS USING ENZYMATIC OXYGEN CONSUMPTION IN THE HEMOX-ANALYZER

V.W. Macdonald and Suneel Bhat. Walter Reed Army Institute of Research, Silver Spring, MD 20910

A rapid method for measurement of hemoglobin-oxygen equilibrium curves using enzymatic oxygen depletion (Vandegriff, K.D. et al., Analytical Biochemistry 256:107-116, 1998) was adapted for use in the Hemox Analyzer (TCS Scientific). The conversion of protocatechuic acid to 3-carboxy-*cis,cis*-muconate by protocatechuate 3,4-dioxygenase was an effective oxygen scavenging reaction in this instrument with several buffer formulations. A slow nitrogen gas stream into the top gas phase of the cuvette had to be provided in order to achieve oxygen partial pressures below 4 Torr. This system of oxygen removal eliminates the need for potentially destructive bubbling of protein solutions with nitrogen and oxygen and provides complete curve generation in less than four minutes. A new PC data acquisition interface was assembled (Keithley Instruments, Inc.) and software written (LabVIEW™, National Instruments) that simplifies calibration procedures and passes variable array data directly into an array processing software package (MATLAB®, The Mathworks, Inc.) for customized graphing and mathematical analysis. Several chemically-modified human hemoglobins, HbAo, and myoglobin have been characterized with this system using both bis-Tris and bis-Tris propane buffers (pH 7.40). The enzymatic reaction was also run in Hemox-Solution, which is a balanced salt solution containing imidazole buffer and glucose. Curves generated in this manner with fresh blood were typical of those reported in the literature for this instrument using standard gas bubbling techniques. In addition to avoiding problems of gas-phase mediated hemoglobin oxidation, the method appears well-suited for measurements of steady-state oxygen binding curves of red blood cells that may possess pathophysiological, age-related, or species-specific fragility.

PO-24. CELL-FREE HEMOGLOBIN DIFFUSION: IMPLICATIONS FOR THE DESIGN OF HEMOGLOBIN-BASED OXYGEN CARRIERS

M.R. McCarthy¹, K.D. Vandegriff², and R.M. Winslow². ¹University of California, San Diego, CA 92161, ²Sangart, Inc., San Diego, CA 92121

We compared rates of oxygen transport in an *in vitro* capillary system using red blood cells (RBCs) compared to cell-free hemoglobin solutions. The PO₂ drop down the axial length of the capillary was calculated using finite element analysis to match the calculated and measured PO₂ at the exit of the capillary. Human RBCs, purified hemoglobin (HbA₀), hemoglobin cross-linked between \forall Lys99 subunits ($\forall\forall$ -Hb), and bovine hemoglobin conjugated to polyethylene-glycol (PEG-Hb) were evaluated. PEG-Hb showed the least desaturation down the capillary, which closely paralleled the RBC profile. In contrast, HbA₀ and $\forall\forall$ -Hb showed much greater desaturation in the capillary. The hemoglobin diffusion rates are consistent with the Stokes-Einstein Law, showing an inverse relation between the macromolecular diffusion constant and solution viscosity and/or molecular size. The *in vitro* rate of O₂ transport correlated with blood pressure effects in rats following exchange transfusion with cell-free hemoglobins: $\forall\forall$ -Hb \exists HbA₀ \gg PEG-Hb. Neither RBCs nor PEG-Hb infusion increased blood pressure. These results support the hypothesis that cell-free hemoglobin-induced vasoconstriction is mediated by oxygen-sensing regulation of blood flow.

PO-25. THE EFFECTS OF SURFACE MODIFICATION OF HEMOGLOBIN VESICLES AND INHIBITION OF PLATELET ACTIVATION BY SYNTHETIC LIPIDS

K. Mori¹, K. Sou¹, S. Takeoka¹, Kazuhiro Suganuma², Makoto Suematsu², and E. Tsuchida¹

¹Dept. of Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555, Japan

²Keio University School of Medicine, Tokyo 160-8582, Japan

We have tried to determine the optimum lipid composition of hemoglobin vesicles (HbV) which have the cellular structure of phospholipid vesicles containing concentrated Hb. Poly(ethylene glycol)(PEG)-lipid is used for the surface modification of HbV in order to stabilize the dispersion state and to prolong the blood circulation. We studied about the effect of surface modification using synthetic PEG-lipids, which have the various molecular weights of the PEG part and the various numbers of the acyl chains¹. We have also explored synthetic negatively charged lipids as substitutes of dipalmitoyl-phosphatidylglycerol(DPPG) known to activate rat's platelet.

We synthesized a series of PEG-lipids using lysine monodendron as a connector. Stability of the PEG-modified vesicles was measured from the change of the incorporated amount of the PEG-lipids by ¹H-NMR. The vesicles containing a synthetic negatively charged lipid were intravenously injected into the Wistar rat, and the platelet count was measured at 40 min post-injection.

An increased number of acyl chains resulted in the tight immobilization of the PEG-lipids having long PEG chain onto the vesicular surface. The surface modification with the long PEG chains significantly increased the dispersion stability of the vesicles. Therefore, it is expected to prolong the circulation time of the HbV modified with the long PEG chain. Vesicles containing the synthetic negatively charged lipid was prepared in the same way as those containing PG. Striking decline in platelet count observed in the rat after the injection of the vesicles containing PO was not observed in the case of the synthetic negatively charged lipid. Therefore, it is possible to construct more efficient lipid composition of HbV using these easily synthesized PEG- and negatively charged lipids.

Reference

1) S. Takeoka et al. J. Am. Chem. Soc. 122:7927-7935, 2000.

PO-26. REPARATION AND CHARACTERIZATION OF S-NITROSYLATED AND POLYETHYLENE GLYCOL-MODIFIED HEMOGLOBIN AS A CANDIDATE OXYGEN TRANSPORTING CARRIER

K. Nakai¹, H. Togashi², I. Sakuma³, T. Yasukohchi⁴, S. Fujii³, M. Yoshioka², H. Satoh¹, and A. Kitabatake³. ¹Environmental Health Science, Tohoku University Graduate School of Medicine, Sendai, Departments of ²Pharmacology and ³Cardiovascular Medicine, Hokkaido Univ. Graduate School of Medicine, Sapporo, ⁴Oleo Chemistry Research Laboratory, NOF Co., Kawasaki, Japan

Acellular hemoglobin (Hb) derivatives developed as red blood cell substitutes are known to be potent vasoconstrictors due to their EDRF/NO scavenging action. Recently, S-nitrosylated Hb has been reported to act as an NO-donor. We therefore developed s-nitrosylated polyethylene glycol-modified Hb (SNO-PEG-Hb) derivatives as a new candidate oxygen carrier. S-nitrosylation of Hb previously modified with PEGs of various molecular weights was achieved by addition of s-nitrosoglutathione at pH 8.6 for 10 hours at 4 degree C. The molecular weights and s-nitrosylation contents were calculated with HPLC methods. The final products were dialyzed to saline and frozen until use. Their vascular effects were examined by intravenous (i.v.) injection of the products (125 mg Hb/kg) to halothane-anesthetized rats. SH residues of PEG-Hb were successfully s-nitrosylated by 30-70% depending on the amount of s-nitrosoglutathione, and the decomposition of SNO-PEG-Hb was not noted during dialysis and storage. I.v. injection of unmodified Hb to the rats increased arterial blood pressure, while SNO-PEG-Hb did not exert a significant pressor effect. We will also present several other *in vivo* characteristics of SNO-PEG-Hb. It is suggested that SNO-PEG-Hb possesses desirable properties as a material for Hb-based oxygen carrier.

PO-27. LARGE PARTICLE FRACTION CONTRIBUTES TO PFC EMULSION INTERFERENCE OF AUTOMATED BLOOD CELL ANALYSIS: LACK OF EFFECT WITH *OXYGENT*TM

V.V. Obraztsov, and C.M. Woods. Department of Biological Research, Alliance Pharmaceutical Corp., San Diego, CA 92121

The presence of a perfluorochemical emulsion (PFE) in blood has been reported to give spurious elevations in white blood cell and platelet counts with automated blood cell analyzers (Cuignet O.Y. et al Anesth. Analg. 2000, 90:517). To assess the mechanism of this observation, we used two PFEs, *Oxygent* (Alliance Pharmaceutical Corp.) and an experimental PFE (with the same PFC composition as *Oxygent* but with larger median particle size). These were mixed with human blood and blood cells were counted using a Baker Diagnostics System 9000 (Serono-Baker Diagnostic Inc., Allentown, PA), an electroimpedence-type analyzer. Particle size distribution was measured by photosedimentation. Median particle diameters of *Oxygent* and the experimental PFE were $0.18 \pm 0.12 \mu\text{m}$ and $0.30 \pm 0.20 \mu\text{m}$, respectively. At concentrations up to 20%, *Oxygent* had no effect on blood cell count. In contrast, the larger particle sized formulation did cause an apparent increase in both WBC and platelet count.

Blood mixed with:		WBC $\times 10^3/\mu\text{l}$	RBC $\times 10^6/\mu\text{l}$	Platelets $\times 10^3/\mu\text{l}$
10 %	Control (PBS)	5.4 ± 0.2	4.47 ± 0.12	224 ± 9
	<i>Oxygent</i> TM	5.4 ± 0.3	4.38 ± 0.26	228 ± 18
	Experimental PFE	9.2 ± 0.3	4.47 ± 0.09	307 ± 7
20 %	Control (PBS)	4.8 ± 0.1	3.92 ± 0.04	208 ± 8
	<i>Oxygent</i> TM	4.9 ± 0.1	3.89 ± 0.09	206 ± 6
	Experimental PFE	14.4 ± 0.4	3.96 ± 0.05	347 ± 9

Particle size distribution analysis of the two emulsions revealed that *Oxygent* had no particles with diameter $>0.8 \mu\text{m}$ while the experimental PFE had 4.7% $>0.8 \mu\text{m}$ particles. Filtration of the experimental PFE through different pore-sized filters confirmed that it is the large particle ($>0.8 \mu\text{m}$) content in PFEs that is responsible for spurious readings in WBC and platelet count measurements.

PO-28. PHOTOREDUCTION OF METHb BY IRRADIATION IN NEAR-ULTRAVIOLET REGION¹⁾

H. Onuma, H. Sakai, M. Umeyama, S. Takeoka, and E. Tsuchida. Department of Polymer Chemistry, Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan

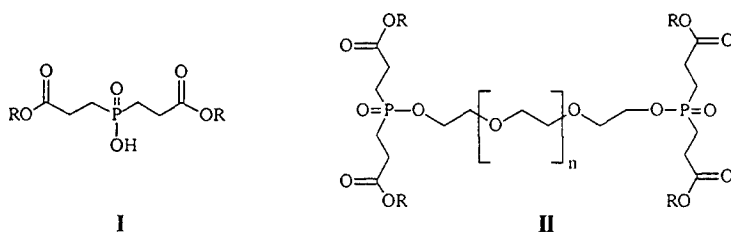
Ferric metHb can be photoreduced to ferrous state by direct photo-excitation in near-ultraviolet region. In this research, we studied the mechanism and facilitating conditions for the photoreduction and the resulting restoration of O₂ binding. MetHb in phosphate buffered saline or pure water in a CO atmosphere was photoreduced to form HbCO by illuminating the *N* band (365 nm), one of the porphyrin (π , π^*) transitions, whereas the photoreduction did not occur in Ar, N₂, or O₂. The transient absorption spectrum detected the generation of deoxyHb within 30 ns in both the CO and Ar atmospheres, however, only in CO did the subsequent CO binding inhibit the back reaction. The photoreduction rate was dependent on the pH and ligand anions, showing that aquametHb in the high spin state was predominant for the photoreduction. Axial ligand-to-metal charge transfer (LMCT) bands overlap with the Soret and *Q* bands in metHb, however, the excitation of these bands showed little photoreduction, indicating that the contribution of these LMCT bands is minimal. Excitation of the *N* band significantly contributes to the photoreduction, and this is facilitated by the external addition of mannitol, hyaluronic acid, Trp, Tyr, *etc.* Especially, Trp allowed the photoreduction even in an Ar atmosphere, and the reduced Hb can be converted to HbO₂ by O₂ bubbling. One mechanism of the metHb photoreduction that is proposed based on these results consists of a charge transfer from the porphyrin ring to the central ferric iron to form the porphyrin π cation radical and ferrous iron by the *N* band excitation, and the contribution of the amino acid residues in the globin chain as an electron donor or an electron pathway.

1) H. Sakai, H. Onuma, M. Umeyama, S. Takeoka, and E. Tsuchida. *Biochemistry* 2000; 39 (in press).

PO-29. DESIGN AND SYNTHESIS OF NOVEL BIFUNCTIONAL AND POLYFUNCTIONAL ORGANIC REAGENTS BASED ON 3-[(2-CARBOXYETHYL)(HYDROXY)PHOSPHINOYL]PROPA-NOIC ACID, AND PROGRESS TOWARD CROSS-LINKING OF HEMOGLOBIN AND STUDIES OF OXYGEN EQUILIBRIA OF MODIFIED HEMOGLOBIN PRODUCTS

T.A. Roach¹, R.S. Hosmane¹, and V.W. Macdonald². ¹Laboratory for Drug Design and Synthesis, Department of Chemistry & Biochemistry, University of Maryland, Baltimore County, Baltimore, MD 21250, ²Blood Research Detachment, Walter Reed Army Institute of Research, Washington, D. C. 20910

One of our current research thrusts is to design and synthesize novel bifunctional organic reagents (BORs), such as I, that are specific for cross-linking hemoglobin at the β -cleft site, as well as polyfunctional organic reagents (PORs), such as II, that are capable of simultaneous cross-linking and oligomerization. Both I and II incorporate multiple



ester functionalities for formation of cross-links with the amino groups of various lysine residues of the β -subunits of hemoglobin, while their inherent anionic charges that are incorporated in the central phosphinic acid hydroxyl and/or the terminal R groups would afford the necessary electrostatic affinity for the β -cleft of hemoglobin. Reagent I was synthesized by DCC coupling of 3-[(2-carboxyethyl)(hydroxy)phosphinoyl]propanoic acid with the appropriately substituted benzene, phenol, or phosphoric acid derivatives. Reagent II was prepared by Mitsunobu condensation of I with the appropriate oligoethylene glycol. Our progress to-date toward hemoglobin cross-linking and assessment of oxygen affinity of modified Hb products will also be reported.

PO-30. INFLUENCE OF Hb-VESICLES ON PHAGOCYtic ACTIVITY AND HISTOPATHOLOGICAL EXAMINATION OF METABOLISM.

H. Sakai¹, H. Horinouchi², K. Tomiyama¹, E. Ikeda², S. Takeoka¹, K. Kobayashi², and E. Tsuchida¹. ¹Department of Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555, ²Department of Surgery & Pathology, Keio University School of Medicine, Tokyo 160-8582, Japan

The influence of Hb-vesicles (HbV) on reticuloendothelial system (RES) was studied by carbon clearance measurement and histopathological examination. HbV suspension was infused intravenously in male Wistar rats at dose rates of 10 and 20 ml/kg, and phagocytic activity was measured by monitoring the rate of carbon clearance at 8 hrs, and 1, 3, 7 and 14 days after infusion. Phagocytic activity decreased transiently one day after infusion by about 40%, and it recovered and enhanced at 3 days, showing a maximum of about twice of the original level at 7 days, and then returned to the original level at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. The succeeding increased phagocytic activity corresponds to the increased metabolism of HbV. The histopathological examination with hematoxylin/eosin and anti-human Hb antibody staining showed that HbV was metabolized within 7 days. Hemosiderin was slightly confirmed with Berlin blue staining at 3 and 7 days in liver and spleen, though they disappeared at 14 days, indicating that the heme metabolism, excretion or recycling of iron ion proceeded smoothly and siderosis was minimal. Electron microscopic examination of spleen and liver tissue clearly demonstrated the vesicular structure of HbV with diameter of about 1/40 of RBC in capillaries, and in phagosomes as entrapped in spleen macrophages and Kupffer cells at one day after infusion. The vesicular structure could not be observed at 7 days. Even though infusion of HbV modified phagocytic activity for two weeks, it does not seem to cause any irreversible damage to the phagocytic organs. These results offer important information for evaluating the safety of HbV for the clinical use.

PO-31. FREE HEMOGLOBIN (Hb) MEDIATES THE SYNTHESIS OF 8-ISO PROSTAGLANDIN F_{2α}, A VASOCONSTRICTIVE ISOPROSTANE

J. Simoni¹, G. Simoni¹, D.E. Wesson², J.A. Griswold¹, and M. Feola¹. Departments of ¹Surgery and ²Internal Medicine, Texas Tech University HSC, School of Medicine, Lubbock, TX 79430

Despite significant progress in the understanding of Hb's intrinsic toxicity, many of its aspects still await full elucidation. We disputed whether other factors, aside from the scavenging of nitric oxide (NO) by Hb, may contribute to Hb-mediated vasoconstriction. The results of our earlier experiments with human endothelial cells incubated with native Hb cast doubt on the role of Hb in the production of thromboxane A₂ and questioned its direct involvement in augmentation of endothelin-1 synthesis. Instead, these experiments evidenced the formation of vasoconstrictive isoprostane 8-iso prostaglandin F_{2α} (8-iso PGF_{2α}). This is an F₂-isoprostane formed *via* a non-cyclooxygenase pathway under the condition of oxidative stress through free radical action on arachidonic acid in cell membranes. This isoprostane is a potent vasoconstrictor, particularly in pulmonary and renal arteries, acting *via* a thromboxane receptor. In addition, subthreshold concentrations of 8-iso PGF_{2α} enhance the vasoconstriction induced by angiotensin II. It was also reported that a lack of NO may accelerate 8-iso PGF_{2α} vasoconstrictor activity. In the present study, we investigated the actual Hb contribution to 8-iso PGF_{2α} synthesis in buffered arachidonic acid, whole human blood and plasma, using tetrameric Hbs with different iron oxidative status and with and without presence of hydrogen peroxide (H₂O₂): 1) ferrous-Hb, 2) ferric-Hb, and 3) ferryl-Hb. This study showed that ferrous-Hb can easily convert arachidonic acid into 8-iso PGF_{2α} and that this effect is correlated with Hb's oxidation rate, and is time and dose dependent. The conversion of arachidonic acid into 8-iso PGF_{2α} by ferryl- and ferric-Hb was also effective. The presence of H₂O₂ in Hb solutions accelerated this reaction. Similar relations were found when human blood and plasma were exposed to these Hbs, however, whole blood generated more 8-iso PGF_{2α} than plasma alone. These data suggest: 1) the redox cycling between different oxidation states of Hb is a primary factor responsible for formation of this potent vasoconstrictor isoprostane, and 2) 8-iso PGF_{2α} is a factor in the not yet fully understood Hb-mediated vasoconstriction. These results also imply that the monitoring of plasma (or urine) concentrations of isoprostanes after the administration of Hb-based blood substitutes could be a useful test in the estimation of oxidative stress *in vivo*.

PO-32. A NOVEL HEMOGLOBIN-ADENOSINE-GLUTATHIONE BASED RED CELL SUBSTITUTE: EVALUATION OF ITS EFFECTS ON HUMAN BLOOD *EX VIVO*

J. Simoni¹, G. Simoni¹, D.E. Wesson², J.A. Griswold¹, and M. Feola¹. Departments of ¹Surgery and ²Internal Medicine, Texas Tech University HSC, School of Medicine, Lubbock, TX 79430

Chemically modified hemoglobin (Hb) solutions are under current investigation as potential blood substitutes. We have developed a novel Hb-based blood substitute composed of purified bovine Hb cross-linked intramolecularly with *o*-ATP and intermolecularly with *o*-adenosine, and conjugated with reduced glutathione (GSH). In this study, we compared the effects of our novel blood substitute and those of unmodified (U) Hb, using allogenic plasma as a control, on human red blood cells (RBC), platelets, monocytes (Mo), and low-density lipoproteins (LDL). The pro-oxidant potential of both Hb solutions on RBC's was examined by the measurement of osmotic and mechanical fragility, conjugated dienes (CD), lipid hydroperoxides (LOOH), thiobarbituric acid reactants (TBAR-S), isoprostanes (8-iso PGF_{2α}) and intracellular GSH. The oxidative modification of LDL was assessed by CD, LOOH and TBAR-S. The effect of Hb on platelets was studied by monitoring their responses to the aggregation agonists: collagen, ADP, epinephrine and arachidonic acid. Monocytes were cultured with Hb solutions or plasma and tested for TNFα and IL-1β release and examined by electron microscopy. Results indicate that native UHb initiates oxidative stress to many blood components and stimulates inflammatory responses by Mo. It caused an increase in RBC osmotic and mechanical fragility ($p < 0.001$). While the level of GSH was slightly changed, the lipid peroxidation of RBC increased ($p < 0.001$). UHb was found to be a stimulator of 8-iso PGF_{2α} synthesis, a potent oxidizer of LDL, and an effective potentiator of agonist-induced platelet aggregation. Contrarily, our novel blood substitute did not appear to induce oxidative stress nor to increase Mo inflammatory reactions. The osmotic and mechanical fragility of RBC was similar to that of the control. Such modified Hb failed to oxidize LDL, or to increase the production of 8-iso PGF_{2α}. It markedly inhibited platelet aggregation. The effect of this novel blood substitute can be linked with the pharmacological properties of adenosine and GSH, which are used as a cross-linker and surface modifier, and the type of modification procedure that lowers the Hb pro-oxidant potential.

PO-33. OXIDATIVE REACTIONS OF HEMOGLOBIN (Hb) CROSS-LINKED WITH ADENOSINE AND CONJUGATED WITH REDUCED GLUTATHIONE (GSH)

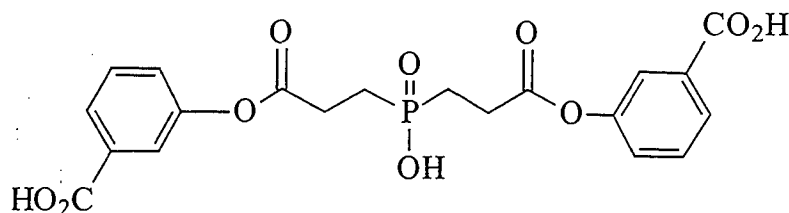
J. Simoni¹, G. Simoni¹, D.E. Wesson², J.A. Griswold¹, and M. Feola¹. Departments of ¹Surgery and ²Internal Medicine, Texas Tech University HSC, School of Medicine, Lubbock, TX 79430

Free Hb is a source of free radicals and is highly reactive with peroxides. The autoxidation of Hb generates a superoxide anion, which can be a source of hydrogen peroxide (H_2O_2) and hydroxyl radicals. The reaction between ferric Hb and H_2O_2 produces two toxic intermediates, a ferryl heme iron ($Fe^{+4}=O$) and a globin tyrosyl radical ($gl(tyr^*)Fe^{+4}$). It is believed that these reactive species may be formed after administration of free Hb-based oxygen carriers, especially in ischemic patients with diminished ability to control oxidative reactions. In this study, we have evaluated the pro-oxidant potential of our Hb-based blood substitute consisting of bovine Hb cross-linked intramolecularly with *o*-ATP and intermolecularly with *o*-adenosine and conjugated with GSH, using bovine unmodified, tetrameric Hb (UHb) as the control. *In vitro* assessment of Hb pro-oxidant potential was done by monitoring: 1) autoxidation rate, 2) pseudoperoxidase-like activity, 3) formation of $Fe^{+4}=O$, and 4) formation of $gl(tyr^*)Fe^{+4}$. All reactions were conducted in 50 mM phosphate buffer, pH 7.0, at 37°C using, except for the autoxidation study, a 2.5 molar excess of H_2O_2 with respect to heme. The Hb autoxidation rate and the formation of $Fe^{+4}=O$ were detected by spectrophotometry, using reported extinction coefficients. The Hb's pseudoperoxidase-like activity was evaluated by spectrophotometrical assessment of H_2O_2 -dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB). The formation of $gl(tyr^*)Fe^{+4}$ was measured indirectly by electrophoretic analysis of structural modification of apolipoprotein (apo) B of human low density lipoproteins (LDL). Results indicate that UHb has a high autoxidation rate and pseudoperoxidase-like activity. UHb when reacted with a 2.5-fold excess of H_2O_2 can easily be converted into both, $Fe^{+4}=O$ and $gl(tyr^*)Fe^{+4}$. On the contrary, the blood substitute autoxidation rate, TMB oxidation, and the formation of $Fe^{+4}=O$ were lower than that of UHb, by 28%, 10%, and 43%, respectively. This Hb solution in the presence of H_2O_2 failed to modify apo B, which is indicative of a lack of $gl(tyr^*)Fe^{+4}$ formation. These data reveal that significant reduction of the natural pro-oxidant potential of Hb can be achieved by selective targeting of amino acid residues of Hb β chains with *o*-adenosine and incorporation of GSH.

PO-34. SYNTHESIS, HEMOGLOBIN CROSS-LINKING, AND ASSESSMENT OF OXYGEN AFFINITY OF MODIFIED HEMOGLOBIN PRODUCTS OF NOVEL BIFUNCTIONAL ORGANIC REAGENT, BIS[2-(3-CARBOXYPHENOXY)- CARBONYLETHYL]PHOSPHINIC ACID (*m*-BCCEP)

K.S. Somerville-Armstrong¹, R.S. Hosmane¹, and V.W. Macdonald². ¹Laboratory for Drug Design and Synthesis, Department of Chemistry & Biochemistry, University of Maryland, Baltimore County, Baltimore, MD 21250, ²Blood Research Detachment, Walter Reed Army Institute of Research, Washington, D. C. 20910

The synthesis, hemoglobin cross-linking, and oxygen equilibrium studies of modified hemoglobin products of a novel bifunctional organic reagent, bis[2-(3-carboxyphenoxy)carbonylethyl]phosphinic acid (*m*-BCCEP) will be reported. The reagent was synthesized in four



***m*-BCCEP**

easy steps starting from *m*-hydroxybenzoic acid. The tri-sodium salt of *m*-BCCEP was employed to cross-link oxyHb, and the product was purified by DEAE-cellulose chromatography. The purified material was analyzed by SDS-PAGE, IEF, and HPLC analyses, which clearly showed the formation of covalent, intramolecular cross-links. While SDS-PAGE analyses of individual bands pointed to the molecular weight range of 32 kDa, the HPLC analyses suggested that the cross-links had formed between β_1 - β_2 subunits. The details of oxygen equilibrium measurements, performed on the purified bands of the modified hemoglobins, will also be presented.

PO-35. SURFACE MODIFICATION OF THE HEMOGLOBIN-VESICLES BY SPONTANEOUS INCORPORATION OF THE POLY(ETHYLENEGLYCOL)-LIPIDS

K. Sou, T. Endo, S. Takeoka, and E. Tsuchida. Department of Polymer Chemistry, Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan

The phospholipid vesicles encapsulated hemoglobin (HbV) has been studied as an oxygen infusion. The poly(ethyleneglycol)-lipid (PEG-lipid) is extensively used to modify the surface of the vesicles for the prevention of the their aggregation and prolongation of the blood circulation time of the vesicles by preventing the uptake by reticuloendothelial systems (RES). When the PEG-lipid solution was added to the vesicle dispersion, the PEG-lipids was spontaneously incorporated into the outer surface of the vesicles, and the surface of the vesicles is modified with the PEG chains.

The physicochemical parameters such as rates of incorporation and dissociation (k_{on} , k_{off}), enthalpy change (ΔH), and equilibrium constant (K) were measured with a fluorescent indicator, an isothermal titration microcalorimeter (ITC), and a 1H -NMR. The dispersion stability of the surface-modified vesicles prepared by this spontaneous incorporation was analyzed from the critical molecular weight of a polymer for the aggregation of the vesicles.

The PEG-lipid incorporated into vesicles in a monomer state, and the K values of the PEG-lipid incorporated into vesicles increased with the alkyl chain length of both PEG-lipids and vesicular components because of the significant decrease of the k_{off} , even if the k_{on} was decreased. The aggregation of the vesicles was successfully suppressed with an increase in the molecular weight of the PEG in the PEG-lipid and its incorporation ratio. The ideal PEG-lipid for the surface modification is not only high efficacy, but also high incorporation stability to prevent the dissociation of the PEG-lipid during the circulation. The one of the possible approach to develop these two requirements simultaneously is enlargement of both PEG and hydrophobic part correspondingly. We have synthesized such PEG-lipids using the lysine monodendron structure.

Reference

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PO-36. EFFECTS OF GLUTARALDEHYDE POLYMERIZATION ON OXYGEN TRANSPORT AND CYTOTOXICITY OF BOVINE HEMOGLOBIN

A. Summers, F. Wood, Y. Jia, F. D'Agnillo and A.I. Alayash. ¹Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda MD 20892, USA

Oxidative side reactions initiated by either auto-or chemically induced oxidation of Hb can compromise its ability to carry oxygen and may be detrimental to both Hb and tissue. Intra- and intermolecular crosslinking of bovine Hb (HbBv) with glutaraldehyde produces a mixture of low oxygen affinity tetrameric and polymeric species (polyHbBv) that have a lowered oxygen affinity (P_{50}). Under physiological pH (7.4) and chloride concentration (0.15 M), the P_{50} of native Hb and its polymerized form were 27 and 35 mmHg, respectively. Rapid kinetic studies showed greater overall rates of oxygen dissociation (k_{off}) with little or no change in the association of CO (k_{on}) to the modified protein. The pH dependence of the P_{50} (Bohr effect) and cooperativity (n_{max}) were both diminished for polyHbBv by ~ 40% and 54% respectively. At 37°C, both proteins autoxidized in a biphasic fashion with a comparable initial rate, but in the second oxidative phase polyHbBv autoxidized 1.3 times faster than HbBv. However, inclusion of catalase and SOD diminished the buildup of oxygen reactive species causing the second oxidative phase to be slowed down by 50-58% in polyHbBv. Bovine aortic endothelial cells (BAECs) incubated with bovine Hbs and H_2O_2 showed a loss of intracellular glutathione (GSH) which was correlated with the formation of highly reactive ferryl (Fe^{4+}) intermediate. Incubation with Hbs and H_2O_2 also altered cell cycle progression and led to apoptotic cell death. Glutaraldehyde polymerization of HbBv alters its oxygen affinity, autoxidation kinetics and other related redox properties, which may cause greater toxicity when used as an oxygen transport fluid.

PO-37. PEG-CONJUGATION AND DEOXYGENATION ENABLE LONG-TERM PRESERVATION OF Hb-VESICLES AS O₂ CARRIERS IN A LIQUID STATE¹⁾.

S. Takeoka, H. Sakai, K. Tomiyama, K. Sou, and E. Tsuchida. Department of Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555, Japan

The stability of hemoglobin vesicles (HbV) as an oxygen infusion was tested during the storage for two years at 4 °C, 23 °C, and 40 °C. The surface of the HbV was modified with PEG, and the suspension was deoxygenated with nitrogen bubbling.

The samples stored at 4°C and 23°C showed a stable dispersion state for two years, though the sample stored at 40°C degraded; the precipitation and decomposition of vesicular components, a decrease in pH, and 4% leakage of total Hb after one year. The PEG chains on the vesicular surface stabilize the dispersion state and prevent the aggregation and fusion due to their steric hindrance. The original metHb content (*ca.* 3%) before the preservation gradually decreased to less than 1% in all the samples after one month due to the presence of homocysteine inside the vesicles, which consumed the residual oxygen and gradually reduced metHb. The rate of metHb formation was strongly dependent on the partial pressure of oxygen, and no increase in metHb formation was observed due to the intrinsic stability of the deoxygenated Hb. Preservation at 4°C and 23°C slightly reduced P₅₀ (increased the oxygen affinity) from 38 Torr to 36 and 32 Torr, respectively.

Generally, phospholipid vesicles are regarded as unstable capsules; however, the establishment of this pivotal technology will enhance the application of PEG-modified vesicles in other fields. The long-term preservation of oxygen infusion overwhelms the limitation of the blood transfusion system and will contribute to benefiting clinical medicine.

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PO-38. CHARACTERIZATION OF ALBUMIN MICROSPHERES CONJUGATED WITH von WILLEBRAND FACTOR-BINDING DOMAIN OF PLATELET GLYCOPROTEIN Ib α .

Y. Teramura¹, H. Ohkawa¹, S. Takeoka¹, Y. Ikeda², and E. Tsuchida¹. ¹Dept. Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555; ²Keio University School of Medicine, Tokyo 160-8582, Japan

As platelet substitutes, we used albumin microspheres(AMS) as carriers for recognition proteins of platelet membrane because of high biocompatibility, high biodegradability and long clinical experience. One of the recognition proteins we selected was a recombinant fragment of glycoprotein(GP)Ib α of platelet membrane, which recognizes von Willebrand factor(vWf). In this study, we have established the simple method for the preparation of AMS and then rGPIb α -AMS, and evaluated their ability of ristocetin-induced aggregation and the recognition of vWf under flow conditions.

AMS were prepared by pH and temperature control of a recombinant human serum albumin (rHSA) solution. Under the alkaline condition disulfide bonds and one thiol group, which usually exist inside the rHSA, are exposed to the outside, and then the thiol-disulfide exchange reaction occurs intra- and intermolecularly at 80°C. By changing to the neutral pH and 37°C, the AMS grow from 50 to 500nm, resulting from the decrease of the electrostatic repulsion and thiol-disulfide exchange reaction among albumin polymers. We could adjust the diameter of AMS to 240 \pm 10nm. rGPIb α was conjugated to the AMS using N-succinimidyl 3-(2-pyridyldithio) propionate. The number of rGPIb α bound to the AMS surface was controlled from 200 to 2500 molecules per a particle.

By the addition of ristocetin, rGPIb α -AMS specifically aggregated with vWf, and the platelet aggregation was enhanced in a low platelet concentration. Under the flow conditions, rGPIb α -AMS were also specifically attached to the vWf-immobilized surface. Therefore it is considered that rGPIb α -AMS would meet the one of the requirements for platelet substitutes, i.e, recognition ability to vWf under the flow condition.

Reference

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PO-39. EFFECTS OF HEMOGLOBIN DERIVATIVES ON PLATELET FUNCTION IN RATS

H. Togashi¹, K. Nakai², I. Sakuma³, S. Fujii³, M. Yoshioka¹, H. Satoh², and A. Kitabatake³. ¹Department of Pharmacology & ³Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, & ²Department of Environmental Health Science, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan

Acellular hemoglobin (Hb), a candidate for artificial red blood cell substitutes (ARBCSs), possibly stimulates the platelet function due to their EDRF/NO scavenging action. We developed a S-nitrosylated pyridoxalated, long chain polyethylene glycol (PEG)-conjugated Hb (SNO-PEG-Hb), which may act as a NO-donor. The purpose of the present study was to elucidate platelet function of SNO-PEG-Hb in comparison with that of unmodified- and PEG-Hb.

In vitro experiments, dynamic changes in platelet serotonin (5-HT) were evaluated as an index of platelet function. Pool rat plasma was incubated with Hb-derivatives for 10 min at 37 C, and separated into platelet and platelet poor plasma (PPP). Platelet and PPP levels of 5-HT and its major metabolite, 5-hydroxyindole acetic acid (5-HIAA), are measured using HPLC-ECD. To evaluate the function as a NO-donor, oxidative NO metabolites (NOx) were measured using Griess method.

Treatment with Hb derivatives (0.1-1.0%) decreased platelet 5-HT levels and increased PPP 5-HIAA levels in a concentration-dependent manner. However, the response to SNO-PEG-Hb treatment was lesser potent when compared with unmodified- and PEG-Hb treatment. A concomitant increase in PPP NOx levels was also observed after SNO-PEG-Hb treatment.

These findings suggest that SNO-PEG-Hb has desirable properties for a material as Hb-based oxygen carriers.

PO-40. COMPARISON OF THE EFFECTS OF THREE HEMOGLOBIN-BASED OXYGEN CARRYING SOLUTIONS ON NEUTROPHIL AND PLATELET ACTIVATION *IN VITRO*.

Toussaint, M.¹, Latger-Cannard, V.², Caron, A.¹, Lecompte, T.², Menu, P.¹, and Vigneron, C.^{1,2}. ¹Department of Hematology-Physiology, University Henri Poincaré-Nancy 1, 54001 Nancy; ²Department of Biological Hematology, CHU Nancy, 54500 Vandoeuvre les Nancy, France

The aim of this study was to evaluate and to compare the potential effect(s) of three differently modified HBOCs on the activation of polymorphonuclear leukocytes (PMNs) and platelets *in vitro*. The expression of adherence receptors CD62L, CD18 and CD11b, which reflects the activation state of the neutrophils, was assessed by a direct immunofluorescence method using Quantum Simply Cellular® and by flow cytometry analysis on whole human blood incubated with Hb-Dex-BTC, $\alpha\alpha$ -Hb or O-raffinose-poly-Hb at a final concentration of 16 g/L (clinically relevant concentration), for 15, 30 and 60 minutes. The expression of platelet surface antigens GpIb, GpIIb/IIIa, GpIIIa and CD62P was measured on platelet rich plasma (PRP) incubated for 15 minutes with the three HBOCs. Whole blood aggregometry was performed in presence of these solutions after stimulation with collagen (0.5 μ g/mL) and TRAP (Thrombin Receptor Agonist Peptid) (12.5 μ M). The expressions of the three leukocyte adherence receptors, and of the three platelet glycoproteins were similar for each solution and at each time point, to those noted with the reference solution (RPMI medium). Decrease in CD62L and increase in CD18 expression were observed at 15 minutes of incubation but, when compared to the values obtained with TNF α as the positive control of PMN activation, the profile of expression of adherence receptors obtained with HBOC solutions did not conform to the expected activation profile of PMNs. The presence of HBOCs solutions in whole blood did not induce any change in aggregation pattern of platelets in citrated whole blood. The hemoglobin solutions tested whatever the type of chemical modification, did not appear to activate neither PMNs nor platelets *in vitro*.

PO-41. TISSUE PERFUSION DURING EXCHANGE TRANSFUSION: ROLE OF OXYGEN CARRYING CAPACITY

Tsai, A.G. and M. Intaglietta. Dept of Bioengineering; University of California, San Diego; La Jolla, CA 92093-0412

Exchange transfusion with a non-O₂ carrying solution can maintain baseline tissue perfusion and oxygenation until the systemic hematocrit is reduced to 40% of baseline. Exchange transfusion reduces blood viscosity leading to an increased cardiac output, a mechanism that maintains tissue oxygenation despite the reduction in oxygen carrying capacity of blood. In the subcutaneous striated muscle tissue of the hamster skinfold model exchange transfusion was performed with 6% Dextran 70 kDa, a non-O₂ carrying plasma expander (NOC) up to a 60% reduction in systemic hematocrit, maintains tissue oxygenation at baseline levels (23 mm Hg) in the subcutaneous striated muscle tissue of the hamster skinfold chamber, however at 75% reduction in systemic hematocrit, tissue oxygenation was not maintained and capillaries were not perfused. We investigated the use a bovine hemoglobin based oxygen carrying solution (OC) [Oxyglobin, Biopure Inc.] on tissue oxygenation and capillary perfusion as hemodilution was performed beyond the 60% level. Whole blood hemoglobin content after OCS hemodilution was 6.7 g/dl, up from 3.5 g/dl (NOC). Blood pressure was significantly higher at 93% (OC) relative to 62% (NOC). Neither tissue oxygenation nor functional capillary density were improved and remained 10% (2.1 mmHg) and 38% of baseline. Arteriolar and venular diameters were unchanged from baseline, blood flow in these vessels was 50% of baseline, suggesting that larger vessels constricted significantly. In conclusion, restoring whole blood hemoglobin content back to levels which were found to maintain tissue oxygenation with Dextran hemodilution using bovine hemoglobin did not provide an apparent benefit over hemodilution with dextran 70 in terms of hemodynamics and oxygen transport. Findings suggest that O₂ carrying capacity of a replacement solution may not be the primary determinant of tissue perfusion during extreme exchange transfusion.

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PO-42. A COMPUTATIONAL STUDY OF OXYGEN TRANSPORT IN MICROVASCULAR NETWORKS IN THE PRESENCE OF HBOCs

N.M. Tsoukias, A. Vadapalli, D. Goldman, and A.S. Popel. The Johns Hopkins University, Baltimore, MD 21205

A three-dimensional finite difference computational model is utilized to investigate the effects of hemoglobin based oxygen carriers (HBOCs) on tissue oxygenation. The transport of oxygen from a network of capillary vessels to the tissue is simulated in a rectangular region. The capillary network and model's parameters correspond to experimental observations for the hamster cheek-pouch retractor muscle. The blood is treated as a two-phase (plasma/erythrocyte) medium. The spatial distribution of blood flow and hematocrit in the network is considered. The flux of oxygen from each phase is simulated using mass transfer coefficients derived from a detailed finite element analysis of the fluid flow and mass transport in the capillary (A. Vadapalli and A.S. Popel. *Biomaterials Artificial Cells and Immobilization Biotechnology*, 2000). Nonlinear kinetics of oxygen consumption and myoglobin-facilitated diffusion in the tissue are also considered. The spatial distributions of oxygen tension, erythrocytic hemoglobin saturation, and oxygen-carrier saturation are calculated for different HBOCs. The results demonstrate the effect of HBOC's properties such as affinity, cooperativity and concentration on tissue oxygenation.

PO-43. INTRAVASCULAR PERFLUOROCARBON-STABILIZED MICRO-BUBBLES FOR TREATMENT OF HYPOXEMIA DUE TO AN EXPERIMENTAL INTRAPULMONARY SHUNT

Tyssebotn I., Bergoe G., and Lundgren C. Center for Res. and Ed. in Spec. Environments, and Department of Physiology and Biophysics, State University of NY at Buffalo

Circulatory right-to-left shunts present an intriguing treatment problem because O₂ breathing will not substantially increase the O₂ content of the unshunted blood or the total O₂ delivery. Volume-stabilized microbubbles generated by a 2% dodecafluoropentane emulsion (DDFPe) can transport sufficient amounts of O₂ to sustain life in severely anemic O₂-breathing rats (1). Hence, we reasoned that, while increasing the O₂ carrying capacity of the blood by infusion of DDFPe would only marginally increase PaO₂, the total amount of O₂ transported would increase and be reflected in the mixed PvO₂. Three levels of shunting were induced in anesthetized pigs by blocking the airways with steel beads. Group 1 (n=6) shunt fraction was <0.3; Group 2 (n=8) was 0.3-0.5; Group 3 (n=9) >0.5 during O₂ breathing. Treatment was 2% DDFPe, 0.1 ml/kg b.w., in 1-3 i.v. infusions 3-5 hrs apart. Minutes after the infusions started, a rise in PaO₂ was observed in all animals in all groups from 205 to 328 mmHg in Group 1, from 73 to 114 mmHg in Group 2, and from 55 to 69 mmHg in Group 3. The PvO₂ also rose in all three groups and increased on average in all animals by 19±3% (p<0.001) within the first 20 min. PaCO₂ was high and pH was low after shunt application, but gradually normalized after DDFPe infusion. The second and third DDFPe doses induced essentially similar results, but the improvements were more marked and lasted longer (up to 5 hrs). At the end of the experiments, the shunt fractions were unchanged from the air-breathing control values. These results suggest that DDFPe infusion in combination with O₂ breathing could be a valuable treatment to mitigate hypoxemia in right-to-left circulatory shunts of different etiologies.

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PO-44. PREDICTING OXYGEN DISTRIBUTION IN TISSUES IN THE PRESENCE OF HEMOGLOBIN-BASED OXYGEN CARRIERS

A. Vadapalli, and A. S. Popel. Department of Biomedical Engineering, School of Medicine, Johns Hopkins University, MD 21205

The development of hemoglobin-based oxygen carriers (HBOCs) in the last decade has led to many potential clinical applications that include perioperative uses in surgery, resuscitation after traumatic blood loss, and enhancing oxygen delivery to hypoxic tissues. We present a discrete cell mathematical model that predicts the distribution of oxygen under physiological conditions in the presence of HBOCs in the plasma. The concentration of oxygen (O_2) and oxyhemoglobin are determined by solving fundamental fluid flow and mass transport equations using a finite-element numerical method in capillary segments that are representative of the microvasculature and the surrounding tissue cells. We find that the overall conductance to oxygen transport increases with increasing HBOC concentration. For HBOCs with high O_2 affinity the contribution of flux from the RBC decreases towards the venous end of the capillary segment with the opposite being true for HBOCs having low O_2 affinity. We present results for hamster retractor muscle and rat and human myocardium tissue. Supported by NIH Grant HL 18292 and American Heart Association Postdoctoral Fellowship (A.V.).

PO-45. METABOLIC RESPONSES OF CULTURED CELLS TO OXYGENATED PERFLUOROCARBON

J. Wardrop, M.R. Davey, J.B. Power, and K.C. Lowe. School of Life & Environmental Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Inert, respiratory gas-dissolving perfluorochemical (PFC) liquids have been used to augment the supply of oxygen and carbon dioxide to microbial, plant and animal (including human) cells in culture (1). However, there have been few studies on changes in cellular metabolism or on the activities of enzymes responsible for removal of reactive oxygen species during culture of cells in the presence of PFCs. Consequently, protoplast-derived cells of albino *Petunia hybrida* cv. Comanche were used as a model, non-photosynthetic, eukaryotic system to study changes in (i) the rate of oxygen consumption as measured by a Clark-type oxygen microelectrode, (ii) mitochondrial membrane potential (MMP) as assessed by Rhodamine 123 fluorescence, and (iii) intracellular activities of superoxide dismutases (SOD, EC 1.15.1.1) and catalases (CAT, EC 1.11.1.6) following culture for up to 14 d in aqueous nutrient medium overlaying oxygen-gassed perfluorodecalin (*Flutec*® PP5; F2 Chemicals, Preston, UK). The mean (\pm s.e.m., $n = 7$) rate of oxygen consumption of cells after 24 h of culture in the presence of oxygenated PFC was $14.3 \pm 1.6 \mu\text{mol O}_2 \text{ ml}^{-1} \text{ min}^{-1}$, compared to $9.7 \pm 0.8 \mu\text{mol O}_2 \text{ ml}^{-1} \text{ min}^{-1}$ for untreated (control) cells ($P < 0.05$). Similarly, culture of cells with oxygenated PFC for 24 h resulted in a significant ($P < 0.05$) increase of over 50% in the mean MMP, compared to control. Culture of protoplasts with oxygenated PFC also produced significant ($P < 0.05$) increases in both mean SOD and CAT activities after 3-7 d of culture, the former comparable to that reported previously for protoplasts of *Salpiglossis sinuata* cultured with oxygenated PFC (2).

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PO-46. THE PULMONARY MICROCIRCULATION IN LIVING RATS INJECTED RED CELL SUBSTITUTES

T. Yamauchi, M. Yamamoto, T. Otsuka, A. Iwamaru, M. Watanabe, and H. Horinouchi. Koichi Kobayashi, Department of Surgery, School of Medicine, Keio University, Tokyo

The effects of red cell substitutes on the pulmonary microvasculature have not yet been evaluated. Hemoglobin derivatives are considered to work as NO scavenger when administered intravenously. The purpose of this study is to clarify the change of the pulmonary microcirculation of the living rat after injection of various red cell substitutes. [Method] Sprague-Dawley rats weighing 260-280g were anesthetized by halothene, mechanically ventilated, and pulmonary window chamber, which is made of silica glass, was implanted into the right chest wall of rats to allow the observation of the pulmonary microvasculature. The laser confocal microscope was used to observe the lung microcirculation. To visualize the pulmonary vasculature, animals were injected intravenously with fluorescein isothiocyanate-labeled albumin (FITC albumin). We used oxyhemoglobin as red cell substitutes. The animals were divided into the group of oxyhemoglobin injection and that of saline injection. We gave each of them 2ml and measured the diameter of the pulmonary arteriole whose diameter is around 60 μ m. Heart rate was also monitored. [Results] The lung chamber model in rat was found to be valuable for the direct examination of the pulmonary vasculature in vivo. In oxyhemoglobin group the diameter of the pulmonary arteriole changed to 94.8 \pm 7.4% (just after injection), 95.3 \pm 20.5% (5 minutes later), 91.2 \pm 11.2% (60 minutes later). On the other hand, in saline group the diameter of the arteriole changed to 108.4 \pm 14.3% (just after injection), 107.1 \pm 23.5% (5 minutes later), 109.7 \pm 23% (60 minutes later). [Conclusion] Oxyhemoglobin injection lead to diminish the diameter of pulmonary arteriole. These results suggest that oxyhemoglobin may work as NO scavenger in pulmonary microcirculation.

PO-47. PRESERVATION STABILITY OF HUMAN SERUM ALBUMIN-HEME AS AN O₂ INFUSION

T. Yanagimoto, T. Komatsu, and E. Tsuchida. Department of Polymer Chemistry, ARISE, Waseda University, Tokyo 169-8555 Japan

The requirements for O₂ infusion are stability for long-term storage as well as the absence of a blood type antigen and infectious virus. We have recently found that tetrakis(*o*-pivalamido)phenylporphinato-iron(II) derivatives with a covalently linked axial imidazole are efficiently incorporated into recombinant human serum albumin (rHSA), providing a new type of synthetic hemoprotein (rHSA-FeP) which can bind and release O₂ reversibly under physiological condition (phosphate buffer saline, pH 7.4, 37°C) like hemoglobin and myoglobin.^{1,2)} The physicochemical properties and the O₂-transporting ability of rHSA-FeP satisfied the initial clinical requirements for O₂ infusion as a red blood cell substitute.³⁾

We report herein the preservation stability of rHSA-FeP under various conditions. The prepared samples ([rHSA] = 5wt%, FeP/rHSA = 8 (mol/mol)) were stored at 5, 25 and 40°C. The red-colored solution has not been changed for more than two months, and no precipitation was observed. The change in density, viscosity and pH of the solution were negligible, and the isoelectric point of rHSA-FeP was also constant.

The kinetic and equilibrium parameters of O₂ binding to rHSA-FeP with the elapse of storage time are also discussed. The O₂-association and -dissociation rates were determined by the laser flash photolysis. The absorption decays showed three phases of the first order kinetics, which were composed of base elimination, fast O₂ rebinding and slow O₂ rebinding, respectively.²⁾ The further evaluation is now in progress.

1) E. Tsuchida, et al. *Bioconjugate Chem.* 10: 797-802. 1999.

2) T. Komatsu, et al. *Bioconjugate Chem.* 11: in press. 2000.

3) E. Tsuchida, et al. *Bioconjugate Chem.* 11: 46-50. 2000.

PREPARATION OF BLOOD SUBSTITUTE FROM HUMAN PLACENTA BLOOD: A PRELIMINARY STUDY

Hong Wang^{1,2}, Jiabin Liu¹, Min Zeng², Tom T.C.Ruan², Chengmin Yang^{1,2}

¹Institute of Blood Transfusion, CAMS, Chengdu, China, 610081

²Zhuhai. JinYe Scientific and Technological Development Co. Ltd, Zhuhai, China, 19020

The demand for blood transfusion is increasing rapidly because of modern medical treatment, local disaster and military rescue. However, such problems as infectious diseases, immunologic reactions are difficult to overcome. Therefore the research and development (R&D) of blood substitutes have attracted extensive attention around the world. Among which Hb-based red cell substitutes is a major type.

Additionally, similar oxygen-transporting abilities to that of natural human RBCs, Hb-based substitutes possess a higher ability to supply oxygen the microcirculation than that of natural RBCs due to their much smaller particle sizes. Thus as oxygen therapeutic agents do, they are capable of potentially treating of the hypoxic diseases in heart, brain, liver, intestines and muscles, etc., that have been well recognized. It was expected by Dr.F.C.Drees that this kind product can be used as blood substitutes and oxygen therapeutic agents and would display its potentially commercial value of 30 billion USD in America.

In the preparation, of Hb-based substitutes, the source of hemoglobin is a major problem. In China the amount of human out-dated and unqualified blood is a little, because each donor must have passed the medical examination before donation. We have used human placenta blood (HPB) as the source of Hb to produce blood substitute. In China human, placenta blood is abundant. We have designed a patented device to collect effectively human placenta blood. By purification, modification and cross-linking etc., we have got a pyridoxalated poly-Hb from the human placenta Hb, which has no significant difference with that made from adult Hb in physiochemical, physiological and biological, indices (see Table 1).

The results in the animal blood-exchange test also show that there are no obvious differences between the two products made from different sources of Hb (see Table 2). These results suggest that the use of human placenta blood in the R&D of blood substitutes has a very bright future.

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Table 1. Quality comparison of two PP-poly Hb (n=3)

Assays	PP-Poly from HPB	PP-Poly from AB
Hb concentration (g/dl)	8.5 ± 0.3	10.1 ± 0.5
MetHb (%)	4.19 ± 1.3	3.9 ± 0.9
Molecular weight (KD)	120 – 160	64 – 600
Unpolymerized Hb (%)	< 1%	< 5%
PH	7.28 ± 0.2	7.40 ± 0.05
P50 (mmHg)	22.5 ± 1.4	22.5 ± 1.0
COP (mmHg)	20 – 25	20 – 25
Endotoxin	qualified	qualified
Bacteria	qualified	qualified
Pyrogen	negative	negative
Na ⁺ (mEq/L)	not measured	145.0 ± 5.2
K ⁺ (mEq/L)	not measured	4.9 ± 0.3
Cl ⁻ (mEq/L)	not measured	11.36 ± 3.6

Table 2. Results of Blood Exchange Test on rats (n=3)

Sample	Hct		Exchange rate(%)	PO ₂ (mmHg)		24 hour survival
	before	after		before	After	
PP-Poly (HPB)	45.5±3.23	11.7±3.3	74.6±7.1	93.1 ± 13.9	131.0±14.28	3/3
PP-Poly (AB)	46.0±1.46	13.6±10.2	70.7±10.2	87.3 ± 10.0	110.7±32.1	3/3
Control (RL)						0/3

EFFECTS OF MODIFIED HEMOGLOBIN ON MYOCARDIAL MITOCHONDRIA AND SARCOPLASMIC RETICULUM IN VITRO

Min Zeng, C.-M. Yang, Institute of Blood Transfusion, Chinese Academy of Medical Sciences & Peking Union Medical College, P.O.Box 268 Chongdu, P.R of China.
(610081)

In this study, the rabbit myocardial mitochondria and sarcoplasmic reticulum were isolated by differential centrifugation, and observed by transmission electron microscope. Using the rabbit myocardial mitochondria and sarcoplasmic reticulum as experimental material, the effects of modified hemoglobin (pyridoxalated PolyHb) produced in our laboratory on lipid peroxide concentration, membrane fluidity and $^{45}\text{Ca}^{2+}$ uptake were directly investigated in vitro. As a whole, the research results suggested again that there is a close relationship between modified hemoglobin and oxygen free radical generation. It was found that, in rabbit myocardial mitochondria exposed to modified hemoglobin, the lipid peroxide concentration was increased, the membrane fluidity was reduced, whereas the superoxide dismutase was able to highly eliminate these adverse influences. It was also found that, in rabbit myocardial mitochondria and sarcoplasmic reticulum exposed to modified hemoglobin, the $^{45}\text{Ca}^{2+}$ uptake was reduced, whereas the superoxide dismutase was able to obviously increase the $^{45}\text{Ca}^{2+}$ uptake. In conclusion, it is suggested that, one way of the toxic, mechanism of modified hemoglobin must likely be that, modified hemoglobin solutions have a close relationship with the oxygen free radical generation. Furthermore, it could influence biomembrane normal structure and function, could influence the cell regulating ability toward intracellular free calcium concentration, as a result, it may cause sorts of adverse reactions.

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